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Vasilis Vasiliou · Samir Zakhari
Lopa Mishra · Helmut K. Seitz *Editors*

Alcohol and Cancer

Proceedings of the Third International Conference

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Preface

Heavy alcohol consumption is a risk factor for disease and mortality worldwide. According to epidemiological studies, chronic alcohol consumption has also been associated with a variety of cancers (oral cavity, pharynx, larynx, esophagus, liver, colorectum, breast). It appears that the risk of developing cancer increases in proportion to the amount of alcohol consumed. Enigmatically, alcohol intake decreases the risk of thyroid cancer, kidney and lung cancer (with evidence most strongly supporting lower risk for light and moderate drinkers) and non-Hodgkins lymphoma, and does not appear to affect prostate cancer risk. Based upon these epidemiological data, it is evident that the capacity for alcohol to influence carcinogenesis varies between tissues or organs. This being the case, it is not unreasonable to propose that the mechanisms by which alcohol promotes or represses cancer is likely to be tissue-dependent. In addition, individual risk factors including genetics also modify ethanol-mediated carcinogenesis. Given the prevalence of alcohol consumption in societies throughout the world and the challenges associated with effective cancer treatment, a more complete understanding of the risks associated with alcohol exposure use in relation to cancer is particularly important, as is identification of the mechanisms by which alcohol influences cancer development. Recognizing this, the idea for an international meeting that focused on alcohol and cancer was born.

In September 2010, the first International Congress on Alcohol and Cancer was held at the German Cancer Research Center (DKFZ) in Heidelberg, Germany. This meeting provided a venue for the presentation of research that specifically addressed alcohol and cancer. Areas of focus included recent advances in epidemiology, molecular mechanisms and biomarkers of alcohol-induced carcinogenesis, as well as anticancer therapies.

After the success of the first meeting, additional International Congresses on Alcohol and Cancer have been held. The second congress took place in May 2013 at Breckenridge, Colorado, USA. Summaries of presentations from this meeting were published in *Advances of Experimental Medicine and Biology* **815**: 1-436, 2015). In June 2015, the third congress was held in Hersonissos Crete, Greece, in June 2015.

This book comprises papers presented during the third congress. The research described herein documents the significant progress that has been made in our understanding of the molecular mechanisms by which alcohol may affect carcinogenesis, and epidemiological studies examining how alcohol affects the risk of cancer development. We anticipate this book will inform the reader about this important area of alcohol research and stimulate cancer investigators and clinicians to consider how alcohol consumption may affect their research or patient care endeavors. Finally, it is our intention to continue these congresses; the fourth will be held in Newport, Rhode Island, USA, on April 14–18, 2019.

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Chapter 1
Alcohol Consumption and Risk of Thyroid Cancer: A Population Based Case-Control Study in Connecticut

Huang Huang, Nan Zhao, Yingtai Chen, Nicole Deziel, Min Dai, Ni Li, Robert Udelsman, and Yawei Zhang

Abstract

Background: Studies examining the association between alcohol consumption and thyroid cancer risk have been inconsistent, in part due to varying types and amounts of alcohol consumption, incomplete information on confounders, and variations in genetic susceptibility in study populations.

Methods: The present study analyzed data from a population-based case-control study in Connecticut in 2010–2011 including 462 histologically confirmed incident thyroid cancer cases and 498 population-based controls. Unconditional logistic regression was used to estimate associations between alcohol consumption and risk

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of thyroid cancer. Potential confounding variables were age, gender, race, education, body mass index, family history of cancer among first-degree relatives, history of benign thyroid disease, smoking status, and physical activity.

Results: Ever consumption of alcohol was associated with a reduced risk of thyroid cancer (OR = 0.71, 95% CI: 0.54–0.95). The younger age at initiation and increasing duration of alcohol consumption were also associated with a reduced risk of thyroid cancer in a dose-dependent manner (P for trend = 0.041 and 0.0065, respectively). Compared to people who never drank alcohol, people who drank alcohol for >31 years were 50% less likely to develop thyroid cancer (OR = 0.50, 95% CI: 0.32–0.80). Alcohol consumption was associated with a reduced risk of papillary thyroid cancer (OR = 0.66, 95% CI: 0.49–0.88) and thyroid cancer with lager tumor size (>1 cm), but no significant association was found between alcohol consumption and non-papillary thyroid cancer or thyroid microcarcinoma. Analyses stratified by specific subtypes of alcohol demonstrated an inverse association for beer (OR = 0.69, 95% CI: 0.49–0.96) and wine consumption (OR = 0.71, 95% CI: 0.53–0.96) as compared to participants who never consumed alcohol, but no significant association was found for liquor consumption (OR = 0.75, 95% CI: 0.53–1.04).

Conclusions: The study findings suggest an inverse association between alcohol consumption and risk of thyroid cancer. Future mechanistic study is warranted to elucidate the underlying mechanisms.

Keywords Thyroid cancer · Alcohol consumption · Case-control study

1.1 Introduction

Thyroid cancer has the highest prevalence of all endocrine malignancies, and its incidence has been rising faster than any other malignancy in both men and women [5]. In the United States, the incidence rate of thyroid cancer has increased since the early 1980s and most sharply over the past decade, with an average increase of 6.0% per year in men and 6.9% per year in women since 1997 [14]. Some scientists attributed this increased incidence to an increase in the rate of detection of thyroid cancer, especially small papillary carcinomas, by widespread use of ultrasonography [7]. However, studies have reported an increased incidence of thyroid cancer among all tumor sizes in the United States [5, 31], which suggests that over-diagnosis was not the only explanation and that other causes should also be considered [26]. The causal factors underlying thyroid cancer are still poorly understood. The most well-established risk factors for thyroid cancer include increased age, female gender, exposure to ionizing radiation, history of benign thyroid disease, and family history of thyroid cancer [10, 11, 22, 27]. Recent studies have identified higher body weight and height as risk factors for thyroid cancer [13, 23] and other environmental/occupational factors [3, 29].

Alcohol is the world's third common risk factor for disease and disability [4]. Alcohol consumption is associated with an increased risk of numerous cancers, such as liver cancer, breast cancer, and colorectal cancer. However, the direction of observed associations between alcohol consumption and thyroid cancer risk has been inconsistent. Several prospective cohort [1, 18] and case-control studies [8, 16, 24, 25] have shown a statistically significant inverse association between alcohol consumption and risk of thyroid cancer, while others found no significant association [11, 12, 20, 21, 28]. A cross-sectional study which conducted in Korea reported that alcohol consumption was significantly associated with an increased risk of thyroid cancer [6]. The results from a pooled analysis of 10 case-control studies indicated that alcohol consumption was associated with a slightly reduced risk of thyroid cancer [17], but the heterogeneity among the studies was significant. Another pooled analysis showed a significantly stronger association between alcohol consumption and a reduced risk of thyroid cancer among current smokers as compared to former smokers [14]. Many previous studies failed to assess alcohol consumption by different subtypes of alcoholic beverages. Majority of the previous studies collected incomplete information on confounders, such as history of benign thyroid disease and family history of thyroid cancer. Additionally, there were few studies examined the independence of the association between alcohol consumption and thyroid cancer risk with respect to smoking status, which is inversely associated with thyroid cancer.

Since alcohol consumption is one of the commonest modifiable risk factors, it could be of great public health impact to clarify the association between alcohol consumption and thyroid cancer risk. Thus, we analyzed data from a population-based case-control study in Connecticut with detailed information on alcohol consumption and confounding variables. We also investigated the association by smoking status.

1.2 Materials and Methods

The study design has been described previously [3, 30]. Cases were patients with newly diagnosed thyroid cancer between 2010 and 2011 in Connecticut. Eligible cases were residents from Connecticut, aged between 21 and 84 years at diagnosis, had no previous diagnosis of cancer, with the exception of nonmelanoma skin cancer, and were alive at the time of interview. Cases were identified through the Yale Cancer Center's Rapid Case Ascertainment Shared Resource (RCA), the agent of Connecticut Tumor Registry. All cases were histologically confirmed the subtypes of thyroid cancer (papillary, follicular, medullary, and anaplastic). A total of 701 eligible patients with thyroid cancer were identified during the study period. Among them, 462 (65.9%) patients completed in-person interviews and participated in the present study.

Population-based controls were also residents from Connecticut and were recruited using a random digit dialing method. Cases and controls were frequency-matched by age (within ±5 years). A total of 498 individuals participated in the study, with a participation rate of 61.5%.

All study procedures were approved by the Human Investigations Committee at Yale and the Connecticut Department of Public Health. The potential participants were approached by letter and phone. After initial contact, those who agreed to participate were interviewed by trained study interviewers either at the participants' homes or at a convenient location. After obtaining written consent, a standardized and structured questionnaire was used to collect information on alcohol consumption and potential confounding variables.

The participants were asked about alcohol consumption by different types of alcoholic beverages, such as beer, wine, or liquor, over their entire lives. For each type of alcoholic beverage, the participants were asked whether they had ever had at least 12 drinks (yes/no). Exposure to alcohol was defined as ever had at least 12 drinks of any type of alcoholic beverage. For those who had exposed to alcohol, further information on the age at initiation (years), duration (years) and intensity (drinks/month) of alcohol consumption, and lifetime consumption (drinks) was obtained by each type of alcohol.

The frequency distribution of selected characteristics between cases and controls was compared by chi-squared test. Multivariate unconditional logistic regression models were used to estimate odds ratios (ORs) and 95% confidence intervals (95% CIs) for the associations between alcohol consumption and risk of thyroid cancer. We also examined the associations by smoking status (smoker or non-smoker), histological subtype (papillary or non-papillary), tumor size ($\leq$10 mm or > 10 mm), and type of alcoholic beverage (beer, wine, or liquor). The regression models were adjusted for age (continuous), gender, race/ethnicity (whites or others), education (high school or less, technical school or college, graduate school, or others), family history of cancer among first-degree relatives (yes/no), benign thyroid disease (yes/no), body mass index (BMI) (<25, 25–29.9, or $\geq$ 30 kg/m^2), and smoking status. For the analyses stratified by different types of alcoholic beverages, the models were further adjusted for other types of alcohol. All tests were two-sided with $\alpha = 0.05$. All analyses were conducted using SAS software, version 9.3 (SAS Institute, Inc., Cary, North Carolina).

1.3 Results

There were 455 (47.4%) participants who reported that they had ever consumed at least 12 drinks over their entire lives. Among them, 267 (27.8%) had ever consumed at least 12 drinks of beer; 358 (37.3%) had ever consumed at least 12 drinks of wine; and 246 (25.6%) had ever consumed at least 12 drinks of liquor.

The distributions of selected characteristics between thyroid cancer cases and controls were presented in Table 1.1. Cases were younger and less educated, and were more likely to be female, have a family history of thyroid cancer among first-degree relatives, have been diagnosed as benign thyroid disease, and have a higher BMI. Distributions of race/ethnicity, family income, and smoking status were similar among cases and controls.

Table 1.1 Distribution of selected characteristics among thyroid

	Cases (n = 462)		Controls (n = 498)		
	Number	%	Number	%	P
Age (years)					0.0003
<40	86	18.6	64	12.9	
40–49	115	24.9	123	24.7	
50–59	149	32.3	139	27.9	
60–69	81	17.5	100	20.1	
≥70	31	6.7	72	14.5	
Gender					<0.0001
Male	87	18.8	154	30.9	
Female	375	81.2	344	69.1	
Race					0.39
White	415	89.8	450	90.4	
Black	18	3.9	25	5.0	
Other	29	6.3	23	4.6	
Education					0.0021
High school or less	129	27.9	88	17.7	
Technical school or college	216	46.8	261	52.4	
Graduate school	100	21.7	130	26.1	
Other	17	3.7	19	3.8	
Income					0.71
Below poverty level	21	4.6	28	5.6	
Above poverty level	294	63.6	318	63.9	
Confidential or unknown	147	31.8	152	30.5	
Family history of thyroid cancer					0.025
Yes	55	11.9	38	7.6	
No	407	88.1	460	92.4	
Benign thyroid disease					<0.0001
Yes	62	13.4	14	2.8	
No	400	86.6	484	97.2	
BMI (kg/m²)					0.0003
<25	145	31.4	203	40.8	
25-29.9	146	31.6	168	33.7	
≥30	166	35.9	118	23.7	
Missing	5	1.1	9	1.8	
Smoking status					0.18
Smoker	141	30.5	172	34.5	
Non-smoker	321	69.5	326	65.5	

Ever consuming alcohol was associated with a reduced risk of thyroid cancer (OR = 0.71, 95% CI: 0.54–0.95) (Table 1.2). The younger age at initiation and increased duration of alcohol consumption were also associated with a reduced risk of thyroid cancer in a dose-dependent manner (P for trend = 0.041 and 0.0065, respectively). Compared to people who never drank alcohol, people who drank alco-

Table 1.2 Risk of thyroid cancer associated with alcohol consumption

	Cases	Controls	OR[a] (95% CI)
Alcohol			
Never	274	231	1.00
Ever	188	267	**0.71 (0.54–0.95)**
Age at initiation (years)			
<19	71	99	0.72 (0.49–1.06)
19–24	51	80	**0.58 (0.38–0.88)**
>24	64	85	0.84 (0.56–1.26)
P for trend[b]			***0.041***
Duration (years)			
<20	66	76	0.80 (0.52–1.22)
20–31	79	96	0.78 (0.54–1.13)
>31	37	90	**0.50 (0.32–0.80)**
P for trend[b]			***0.0065***
Intensity (drinks/month)			
<28	58	87	**0.59 (0.39–0.89)**
28–60	63	92	0.76 (0.51–1.13)
>60	64	80	0.83 (0.56–1.24)
P for trend[b]			*0.94*
Lifetime consumption (drinks)			
<5200	68	86	0.68 (0.45–1.01)
5200–16,560	52	87	**0.64 (0.42–0.96)**
>16,560	62	84	0.84 (0.56–1.25)
P for trend[b]			*0.55*

[a]Adjusted for age (continuous), gender, race, education, family history of thyroid cancer, history of benign thyroid disease, BMI, and smoking status.
[b]Estimated by continuous variables.

hol for more than 31 years were 50% less likely to develop thyroid cancer (OR = 0.50, 95% CI: 0.32–0.80). People whose age at initiation was between 19 to 24 years were 42% less likely to develop thyroid cancer (OR = 0.58, 95% CI: 0.38–0.88) compared to participants who never drank alcohol. Compared to never drinkers, a reduced thyroid cancer risk was observed among people who drank <28 drinks per month (OR = 0.59, 95% CI: 0.39–0.89) and people whose lifetime consumption was 5200–16,560 drinks (OR = 0.64, 95% CI: 0.42–0.96). However, no statistically significant dose-response relationship was observed for intensity and lifetime alcohol consumption.

The association between alcohol consumption and thyroid cancer risk was modified by smoking status (Table 1.3). A borderline significantly inverse association between alcohol consumption and risk of thyroid cancer was observed among non-smokers (OR = 0.71, 95% CI: 0.50–1.00). This inverse association was even stronger for papillary thyroid cancer among non-smokers (OR = 0.63, 95% CI: 0.44–0.91) (data not shown). However, no significant association was found among smokers. Increasing duration of alcohol consumption was associated with a reduced risk of

Table 1.3 Risk of thyroid cancer associated with alcohol consumption, stratified by smoking status

	Smokers			Non-smokers			
	Cases	Controls	OR[a] (95% CI)	Cases	Controls	OR[a] (95% CI)	P for interaction
Alcohol							0.66
Never	70	66	1.00	204	165	1.00	
Ever	71	106	0.71 (0.43–1.16)	117	161	0.71 (0.50–1.00)	
Age at initiation (years)							0.55
<19	26	30	0.85 (0.43–1.67)	45	69	0.67 (0.41–1.07)	
19–24	19	27	0.72 (0.33–1.54)	32	53	**0.52 (0.31–0.88)**	
>24	25	47	0.61 (0.32–1.16)	39	38	1.04 (0.60–1.79)	
P for trend[b]			0.075			0.22	
Duration (year)							0.85
<20	27	34	0.79 (0.40–1.57)	39	42	0.75 (0.43–1.29)	
20–31	26	37	0.71 (0.37–1.35)	53	59	0.84 (0.53–1.31)	
>31	16	32	0.61 (0.28–1.32)	21	58	**0.45 (0.24–0.82)**	
P for trend[b]			0.19			**0.017**	
Intensity (drink/month)							0.70
<28	26	42	0.54 (0.28–1.06)	32	45	**0.57 (0.33–0.98)**	
28–60	25	40	0.76 (0.39–1.47)	38	52	0.78 (0.47–1.30)	
>60	19	20	1.04 (0.48–2.22)	45	60	0.78 (0.48–1.25)	
P for trend[b]			0.28			0.49	
Lifetime consumption (drink)							0.78
<5200	29	42	0.60 (0.31–1.15)	39	44	0.70 (0.41–1.17)	
5200–16,560	20	37	0.66 (0.32–1.33)	32	50	0.62 (0.37–1.06)	
>16,560	20	21	1.10 (0.52–2.32)	42	63	0.77 (0.47–1.25)	
P for trend[b]			0.53			0.32	

[a]Adjusted for age (continuous), gender, race, education, family history of thyroid cancer, benign thyroid disease, and BMI.

[b]Estimated by continuous variables.

thyroid cancer in a dose-dependent manner among non-smokers (P for trend = 0.017). No statistically significant dose-response relationship was observed among smokers.

As shown in Table 1.4, alcohol consumption was associated with a reduced risk of papillary thyroid cancer (OR = 0.66, 95% CI: 0.49–0.88) and thyroid cancer with lager tumor size (>1 cm) (OR = 0.68, 95% CI: 0.48–0.97), but no significant association was found between alcohol consumption and non-papillary thyroid cancer or thyroid microcarcinoma (≤1 cm).Younger age at initiation and increasing duration of alcohol consumption were associated with a reduced risk of papillary thyroid cancer (P for trend = 0.023 and 0.0016, respectively) and thyroid cancer with lager tumor size (P for trend = 0.036 and 0.035, respectively) in a dose-dependent manner.

When the analyses were stratified by the specific subtypes of alcoholic beverage (Table 1.5), the inversed association between alcohol consumption and risk of thyroid cancer was observed for beer (OR = 0.69, 95% CI: 0.49–0.96) and wine (OR = 0.71, 95% CI: 0.53–0.96), but not liquor (OR = 0.75, 95% CI: 0.53–1.04).

1.4 Discussion

This study found that alcohol consumption was associated with a reduced risk of thyroid cancer. The risk decreased with younger age at initiation and increasing duration of alcohol consumption. The associations were stronger for papillary thyroid cancer and thyroid cancer with larger tumor size. The inverse association between alcohol consumption and risk of thyroid cancer was observed for beer and wine, but not for liquor. The study also observed that while alcohol consumption was associated with a borderline significantly reduced risk of thyroid cancer among non-smokers, no such relationship was seen in smokers.

The underlying mechanism linking alcohol consumption and a reduced risk of thyroid cancer is currently unclear. Zoeller et al. reported a blunting of the thyroid-stimulating hormone (TSH) response to thyrotropin-releasing hormone (TRH) among alcoholics (Zoeller et al. 1996) [32], suggesting that the blunting of TSH response to TRH could lead to a reduction of thyroid cell proliferation and then decrease the risk of thyroid cancer. The mechanism of alcohol-induced reduction in TSH secretion to TRH stimulation is still unclear. One possible explanation is the down-regulation of TRH receptors due to chronically high TRH concentrations. Chronic alcohol consumption is suggested to be associated with a decreased peripheral thyroid hormone levels. This pattern of peripheral low thyroid hormones can chronically induce a slightly elevated TRH release and subsequently cause feedback suppression of the TRH receptors, thereby blunting the downstream TSH secretion [4, 9]. Alcohol is also known to have a direct toxic effect on thyroid cells, which may account for the reduction in the thyroid volume. This toxic effect of alcohol may confer some benefits of thyroid. It has been suggested that alcohol consumption may be protective for the development of goiter and solitary thyroid nodules [4, 15].

Table 1.4 Risk of thyroid cancer associated with alcohol consumption, stratified by subtype of thyroid cancer

		Papillary		Non-papillary		Tumor size ≤1 cm		Tumor size >1 cm	
	Controls	Cases	OR[a] (95% CI)	Cases	OR[a] (95% CI)	Cases	OR[a] (95% CI)	Cases	OR[a] (95% CI)
Alcohol									
Never	231	238	1.00	36	1.00	126	1.00	143	1.00
Ever	267	154	**0.66 (0.49–0.88)**	33	1.10 (0.63–1.91)	91	0.77 (0.54–1.10)	97	**0.68 (0.48–0.97)**
Age at initiation (years)									
<19	99	57	**0.63 (0.42–0.95)**	13	1.32 (0.63–2.76)	30	0.70 (0.42–1.16)	41	0.74 (0.47–1.17)
19–24	80	42	**0.53 (0.34–0.83)**	9	0.99 (0.43–2.28)	25	0.66 (0.38–1.13)	26	**0.54 (0.32–0.92)**
>24	85	53	0.81 (0.53–1.25)	11	1.03 (0.48–2.24)	35	0.96 (0.58–1.58)	29	0.77 (0.46–1.30)
P for trend[b]			**0.023**		0.90		0.32		**0.036**
Duration (year)									
<20	76	57	0.78 (0.50–1.21)	9	1.12 (0.47–2.65)	38	1.07 (0.65–1.78)	28	0.60 (0.35–1.03)
20–31	96	65	0.71 (0.48–1.04)	13	1.16 (0.57–2.39)	34	0.72 (0.44–1.15)	45	0.85 (0.55–1.32)
>31	90	27	**0.42 (0.25–0.70)**	10	1.01 (0.45–2.29)	15	**0.47 (0.24–0.88)**	22	**0.53 (0.30–0.95)**
P for trend[b]			**0.0016**		0.99		**0.045**		**0.035**
Intensity (drink/month)									
<28	87	48	**0.55 (0.36–0.86)**	10	0.93 (0.42–2.05)	28	0.62 (0.37–1.05)	30	**0.59 (0.36–0.98)**
28–60	92	54	0.74 (0.49–1.11)	9	0.91 (0.40–2.06)	33	0.88 (0.54–1.44)	30	0.68 (0.41–1.12)
>60	80	49	0.70 (0.46–1.07)	14	1.72 (0.83–3.57)	28	0.83 (0.49–1.39)	36	0.84 (0.52–1.35)
P for trend[b]			0.64		0.23		0.83		0.93
Lifetime consumption (drink)									
<5200	86	57	**0.63 (0.41–0.97)**	11	1.01 (0.46–2.19)	36	0.81 (0.50–1.33)	32	**0.58 (0.35–0.97)**
5200–16,560	87	43	**0.60 (0.39–0.93)**	9	1.00 (0.44–2.25)	22	0.60 (0.35–1.04)	30	0.69 (0.42–1.14)
>16,560	84	49	0.73 (0.47–1.12)	12	1.39 (0.65–2.98)	29	0.86 (0.51–1.45)	33	0.82 (0.50–1.35)
P for trend[b]			0.24		0.38		0.61		0.65

[a]Adjusted for age (continuous), gender, race, education, family history of thyroid cancer, benign thyroid disease, BMI, and smoking status.
[b]Estimated by continuous variables.

Table 1.5 Risk of thyroid cancer associated with consumption of beer, wine, and liquor

	Beer			Wine			Liquor		
	Cases	Controls	OR[a] (95% CI)	Cases	Controls	OR[a] (95% CI)	Cases	Controls	OR[a] (95% CI)
Alcohol									
Never	274	231	1.00	274	231	1.00	274	231	1.00
Ever	108	159	**0.69 (0.49–0.96)**	149	209	**0.71 (0.53–0.96)**	105	141	0.75 (0.53–1.04)
Age at initiation (years)									
<19	50	78	0.67 (0.43–1.03)	23	28	0.74 (0.40–1.37)	35	41	0.85 (0.51–1.43)
19–24	37	52	0.69 (0.42–1.13)	35	49	0.62 (0.38–1.02)	35	57	**0.55 (0.34–0.90)**
>24	20	29	0.70 (0.36–1.34)	90	131	0.74 (0.52–1.06)	35	43	0.94 (0.56–1.57)
P for trend[b]			0.10			**0.032**			0.32
Duration (year)									
<20	38	53	0.63 (0.38–1.05)	62	77	0.72 (0.47–1.10)	42	42	0.91 (0.55–1.51)
20–31	45	52	0.80 (0.50–1.27)	59	83	0.69 (0.46–1.03)	36	57	**0.57 (0.35–0.92)**
>31	22	51	0.55 (0.30–1.00)	23	46	0.66 (0.37–1.18)	24	41	0.80 (0.45–1.43)
P for trend[b]			**0.037**			**0.039**			**0.036**
Intensity (drink/month)									
<28	60	106	**0.52 (0.35–0.77)**	69	95	**0.67 (0.45–0.98)**	59	88	**0.64 (0.43–0.96)**
28–60	43	41	1.30 (0.77–2.17)	63	87	0.77 (0.52–1.15)	36	42	0.98 (0.59–1.65)
>60	3	8	0.42 (0.10–1.66)	15	22	0.74 (0.36–1.51)	7	6	1.22 (0.38–3.94)
P for trend[b]			0.85			0.21			0.44
Lifetime consumption (drink)									
<5200	56	90	**0.56 (0.37–0.84)**	74	91	0.72 (0.49–1.07)	53	79	**0.62 (0.40–0.94)**
5200–16,560	32	45	0.75 (0.44–1.28)	37	61	0.63 (0.40–1.01)	34	40	0.96 (0.57–1.62)
>16,560	17	18	1.35 (0.65–2.84)	33	50	0.78 (0.47–1.29)	13	17	0.94 (0.43–2.10)
P for trend[b]			0.85			0.10			0.74

[a]Adjusted for age (continuous), gender, race, education, family history of thyroid cancer, benign thyroid disease, BMI, and smoking status.
[b]Estimated by continuous variables.

Several reasons could contribute to the inconsistent results from previous epidemiologic studies. Earlier studies included participants who consumed varying subtypes of alcoholic beverages. A pooled analysis reported a significant trend of decreasing thyroid cancer risk among wine and beer consumers [17]. A population-based case-control study that investigated each alcoholic beverage (beer, wine, and liquor) separately found only wine was associated with a reduced risk of thyroid cancer [16]. Another case-control study selected beer, sake, hard liquor, and whisky as alcoholic beverages and found no significant association [20]. Our study found a reduced risk of thyroid cancer in relation to beer and wine consumption, but the association was non-significant for liquor consumption. Therefore, the selected subtypes of alcoholic beverages and the proportion of each alcoholic beverage among study population may exert an influence on the observed association between alcohol consumption and thyroid cancer risk.

This study also showed a decreased risk of thyroid cancer with younger age at initiation and increasing duration of alcohol consumption. These findings were in accordance with those from several previous studies [1, 14, 16, 18, 20]. However, two prospective cohort studies reported that the frequency and quantity of alcohol consumption was not associated with risk of thyroid cancer [12, 19]. Multiple levels of alcohol consumption and incomplete information on confounders might contribute to the inconsistent results. Kabat et al. modeled the median of each category of exposure as a continuous variable to test the trend [12], while Meinhold et al. modeled categories of exposure as continuous variable to test the trend [19]. Our study used original values of exposure as continuous variable to test the trend. The majority of previous studies was also lacking information on the well-established risk factors for thyroid cancer and did not adjusted for them as potential confounding variables in their models. Adjustment for these risk factors drew the observed association toward the null in our study population. There was only one previous study investigated the association between alcohol consumption and thyroid cancer risk by tumor size and reported an inverse association which was not affected by tumor size [8]. However, alcohol consumption was associated with a reduced risk of thyroid cancer with lager tumor size (>1 cm), but not thyroid microcarcinoma (≤1 cm) in our study.

Given the positive correlation between alcohol consumption and smoking, and the evidence linking smoking with a reduced risk of thyroid cancer [18], residual confounding effect by smoking may bias the association between alcohol consumption and risk of thyroid cancer away from the null. Therefore, we conducted a stratified analysis by smoking status. Our results showed that alcohol consumption was borderline significantly associated with a reduced risk of thyroid cancer among non-smokers, but not smokers.

Aschebrook-Kilfoy et al. found significant interactions between *UGT2B7* and *NAT1* genes and alcohol intake in relation to thyroid cancer risk [2], suggesting that genetic polymorphisms in detoxification genes might modify the relationship between alcohol intake and risk of thyroid cancer. This could also be potential contributor to the inconsistent results from previous studies.

Potential limitations should be considered when interpreting the findings of this study. Information on alcohol consumption was self-reported. Therefore, potential recall bias cannot be ruled out. Additionally, the sample size was limited for more detailed analyses, such as estimation of associations between alcohol consumption and rarer histological subtypes of thyroid cancer, including follicular, medullary, and anaplastic thyroid cancers.

This study also had some strengths. The newly developed thyroid cancer cases could be exhaustively identified by the population-based study design and linkage to the Connecticut Tumor Registry to identify cases. Potential selection bias could be limited. All the cases were histologically confirmed to minimize misclassification of outcomes. Alcohol consumption was assessed by each type of alcoholic beverage. Finally, detailed information on potential confounding factors were collected and controlled for in the analysis.

In conclusion, this study supports the hypothesis that alcohol consumption is associated with a reduced risk of thyroid cancer. The observed protective effect needs to be clarified by more prospective studies. Additional studies investigating the influence of alcohol intake on thyroid hormone and thyroid function could help to improve our understanding of the potential mechanism underlying the inverse association.

Conflicts of Interest none declared

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Chapter 2
Roles of Cytochrome P450 in Metabolism of Ethanol and Carcinogens

F. Peter Guengerich and Narayan G. Avadhani

Abstract Cytochrome P450 (P450) enzymes are involved in the metabolism of carcinogens, as well as drugs, steroids, vitamins, and other classes of chemicals. P450s also oxidize ethanol, in particular P450 2E1. P450 2E1 oxidizes ethanol to acetaldehyde and then to acetic acid, roles also played by alcohol and aldehyde dehydrogenases. The role of P450 2E1 in cancer is complex in that P450 2E1 is also induced by ethanol, P450 2E1 is involved in the bioactivation and detoxication of a number of chemical carcinogens, and ethanol is an inhibitor of P450 2E1. Contrary to some literature, P450 2E1 expression and induction itself does not cause global oxidative stress *in vivo*, as demonstrated in studies using isoniazid treatment and gene deletion studies with rats and mice. However, a major fraction of P450 2E1 is localized in liver mitochondria instead of the endoplasmic reticulum, and studies with site-directed rat P450 2E1 mutants and natural human P450 2E1 N-terminal variants have shown that P450 2E1 localized in mitochondria is catalytically active and more proficient in producing reactive oxygen species and damage. The role of the mitochondrial oxidative stress in ethanol toxicity is still under investigation, as is the mechanism of altered electron transport to P450s that localize inside mitochondria instead of their typical endoplasmic reticulum environment.

Keywords Ethanol · Oxidation by cytochrome P450, in ethanol oxidation · Mitochondria, and cytochrome P450 · Endoplasmic reticulum, and cytochrome P450 · Cytochrome P450 2E1 · P450 2E1 · Enzyme kinetics · Reactive oxygen species · Mitochondrial toxicity · Chemical carcinogens

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2.1 Introduction to Cytochrome P450 (P450)

The field of P450 research stemmed from work in the areas of the metabolism of steroids, drugs, and carcinogens [18, 29]. The human genome contains 57 P450 (*CYP*) genes, of which almost all are expressed in some tissue [29]. The P450s are classified into families and subfamilies on the basis of sequence similarity [52]. However, sequence similarity may or may not be a guide to function with P450s [23, 29].

Another way to classify P450s is by the types of substrates they use (Table 2.1) [29]. Even this approach has caveats in that some P450s could be placed in multiple categories in Table 2.1 (e.g., P450s 1B1 and 27A1) [29]. Another point to make is that several human P450s have yet to have functions identified, i.e. those in the column labeled "unknown." These are often termed the "orphans [30]. However, even when P450s are classified in the "xenobiotics" category and do happen to be able to oxidize a substrate found in the body (e.g. P450 3A4), it is not clear that this reaction has a significant physiological role [29]. In general, the mouse orthologues of this column of P450s can be deleted without profound physiological effects [26].

Collectively the P450s catalyze about 75% of all reactions involved in the metabolism of drugs [81] (some drugs are not readily metabolized, however, and biologicals are not included in this analysis). In the previous analyses [80, 81], the 3A and 2C Subfamily P450s were those most involved in drug metabolism (i.e., 3A4, 2C9,

Table 2.1 Classification of Human P450s Based on Major Substrate Class [29]

Sterols	Xenobiotics	Fatty acids	Eicosanoids	Vitamins	Unknown
1B1[a]	1A1[a]	2J2	4F2	2R1[a]	2A7
7A1[a]	1A2[a]	2U1	4F3	24A1[b]	2S1
7B1	2A6[a]	4A11	4F8	26A1	2 W1
8B1	2A13[a]	4B1[b]	5A1	26B1	4A22
11A1[a]	2B6[a]	4F11	8A1[a]	26C1	4F22
11B1	2C8[a]	4F12		27B1	4X1
11B2	2C9[a]	4V2		27C1	4Z1
17A1[a]	2C18				20A1
19A1[a]	2C19[a]				
21A2[a]	2D6[a]				
27A1	2E1[a]				
39A1	2F1				
46A1[a]	3A4[a]				
51A1[a]	3A5				
	3A7				
	3A43				

[a]X-ray crystal structure(s) reported (for human enzyme)
[b]Rat or rabbit X-ray crystal structure reported

2C19). This pattern still holds for P450s [64]. Further, when one considers oxidation-reduction reactions with all chemicals (for the human enzymes), P450 catalyze 95% of the reactions. Again, the 3A and 2C Subfamily P450s are most prominent [64].

The localization of individual P450s has been reviewed elsewhere [29]. Those P450s involved in critical physiological functions are often found only in a few relevant tissues, e.g. many of the steroidogenic P450s are found only in the adrenals, gonads, etc. Those involved in bile acid processing are generally localized in the liver (e.g. P450 7A1). In general, the xenobiotic-processing P450s are localized principally in the liver, and some are never found outside of the liver, e.g. 1A2, 7A1 [29]. However, some of these are also found at lower levels in many other tissues. Some caution should be used in reviewing the literature, however, in that much of the work is at the mRNA level, not protein.

One of the reasons for the interest in the P450s is their role in the metabolism of toxicants and carcinogens. In general, this process of oxidation (and sometimes reduction) aids in the removal of chemicals from the body and may, along with transporters, be viewed as a mechanism for the body to use a limited set of genes to eliminate natural products that could prove harmful if allowed to accumulate in cells (e.g. alkaloids, terpenes, flavonoids). In general, the oxidation and reduction reactions catalyzed by P450s detoxicate chemicals. However, these same reactions also sometimes create electrophilic species that can react with cellular macromolecules and initiate biological damage, general with DNA or proteins. DNA damage can be misreplicated and fits into the somatic mutation theory of cancer [10]. The relevance of metabolism to chemical carcinogenesis was established largely by the late James and Elizabeth Miller in animal studies [50, 51]. This same concept applies to the toxicity of drugs, with protein damage generally being considered an initial event [24, 37].

Almost all of the information on the roles of human P450s in the metabolism of carcinogens has been published in the past 26 years [72, 73]. One classification indicates that (human) P450s are involved in ~ 2/3 of the reactions in which chemical carcinogens are bioactivated (Fig. 2.1a) [63]. Six human P450s recount for ~ ¾ of these reactions, i.e. P450s 1A1, 1A2, 1B1, 2A6, 2E1, and 3A4 (Fig. 2.1b) [63]. However, P450s are also involved in detoxication of chemical carcinogens [63]. The complexity of these systems can be seen in the classic 1952 Richardson experiment, in which treatment of rats with one carcinogen induced P450s that detoxicate another carcinogen and then had a protective role [65]. We now know that a single P450 catalyze both activation and detoxication of a single carcinogenic chemical, e.g. P450 3A4 oxidizes the hepatocarcinogen aflatoxin B_1 to the 8,9-*exo* epoxide (which reacts efficiently with DNA [36]) and to aflatoxin Q_1 (3α-hydroxylation product), which does not appear to be a carcinogen [77]. This dichotomy appears to be due to the presence of multiple binding modes of the substrate (aflatoxin B_1) in the enzyme active site.

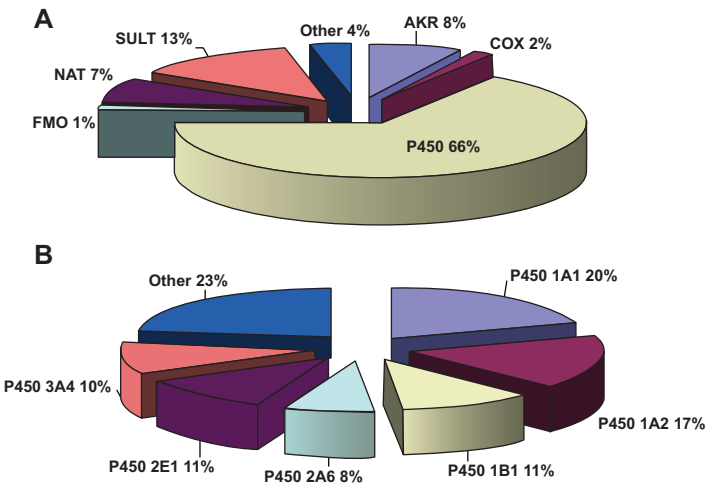

Fig. 2.1 Human enzymes involved in bioactivation of carcinogens [63]. (**a**) fractions of enzyme classes involved in bioactivation; (**b**) individual human P450 contributions to carcinogen activation. *FMO* microsomal flavin-containing monooxygenase, *NAT* N-acetyltransferase, *SULT* sulfotransferase, *AKR* aldo-keto reductase, *COX* cyclooxygenase (prostaglandin synthase)

Table 2.2 Sites of P450 2E1 expression [29]

Liver
Brain
Esophagus
Lung
Nasal mucosa
Pancreas
Small intestine

2.2 P450 2E1

The discovery of P450 2E1 followed largely from work on a microsomal ethanol oxidizing system [47, 57]. P450 2E1 was characterized in rabbit [43] and rat liver [68]. The enzyme is found at highest abundance in liver but also in numerous other tissues of relevance to cancer [25] (Table 2.2).

P450 2E1 is also induced by ethanol [47]. The study of this process has been complicated, in that ethanol also inhibits the enzyme (as expected for a substrate) [82]. Regulation of the CYP2E1 gene is complex and includes both transcriptional and post-transcriptional aspects [25].

P450 2E1 substrates include ethanol, a number of drugs (especially anesthetics), *N*-alkylnitrosamines, vinyl monomers, and halogenated hydrocarbons (Table 2.3) [29, 31]. Several of these are of interest in that a number of the nitrosamines, vinyl monomers, and halogenated hydrocarbons are carcinogens [31]. Nitrosamines are

Table 2.3 Pro-carcinogens and suspected carcinogens known to be bioactivated by P450 2E1 [29, 31, 63]

Acrylonitrile
Aniline
Azoxymethane
Benzene
1,3-Butadiene
Carbon tetrachloride
Chloroform
Cisplatin
1,4-Dichlorobenzene
2,2-Dichloro-1,1,1-trifluoroethane
N,*N*-Diethylnitrosamine
N,*N*-Dimethylformamide
1,2-Dimethylhydrazine
N,*N*-Dimethylnitrosamine
Ethanol
Ethyl carbamate (urethane)
Ethylene dibromide
Ethylene dichloride
Furan
4-Ipomeanol
2-Methoxyaniline
Methylene chloride
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)
N-Nitrosodi-*n*-propylamine
N-Nitrosodiethanolamine
N-Nitrosoethylbutylamine
N-Nitrosomethylbutylamine
N-Nitrosomethylethylamine
N-Nitrosomethylpropylamine
N-Nitrosomorpholine
N-Nitrosopyrrolidine
Nornitrosonicotine (NNN)
Propylene dichloride
Styrene
Trichloroethylene
Vinyl bromide
Vinyl carbamate
Vinyl chloride
4-Vinyl-1-cyclohexene
Vinylidene chloride

present in tobacco, and the vinyl monomers and halogenated hydrocarbons are used on a large scale in industry. These substrates have the general property of small molecular size, consistent with the crystal structure of P450 2E1 [60, 61]. However, it should be pointed out that even fatty acids can be substrates [61]. Because of the induction and inhibition of P450 2E1 by ethanol, the multiples sites of expression of the enzyme (Table 2.2), and the relevance to humans drinking ethanol and exposed to carcinogens in Table 2.2, the co-carcinogenicity of ethanol has been considered in a number of studies with experimental animals [15, 48]. (Co-carcinogenesis studies involve simultaneous administration of chemicals, as opposed to initiation-promotion regimens, with distinct temporal relationships and mechanisms [58]).

2.3 Ethanol Oxidation by P450 2E1

A general view in the alcohol field has been that alcohol dehydrogenase is the most relevant enzyme at low concentrations of ethanol and that P450 (2E1) is more involved at higher concentrations, i.e. those that would be relevant with high consumption. The situation is also complicated in that catalase can also contribute to ethanol metabolism [14, 76].

Even though ethanol has only two carbons and one oxygen, its oxidation has several complexities (Fig. 2.2). The general view in the field is that there are no receptors as such for ethanol, and essentially all of its biological effects are related to acetaldehyde. In aqueous solution, acetaldehyde exists in a roughly equimolar equilibrium with its hydrate, a "*gem* diol" (Fig. 2.2a) [27, 28].

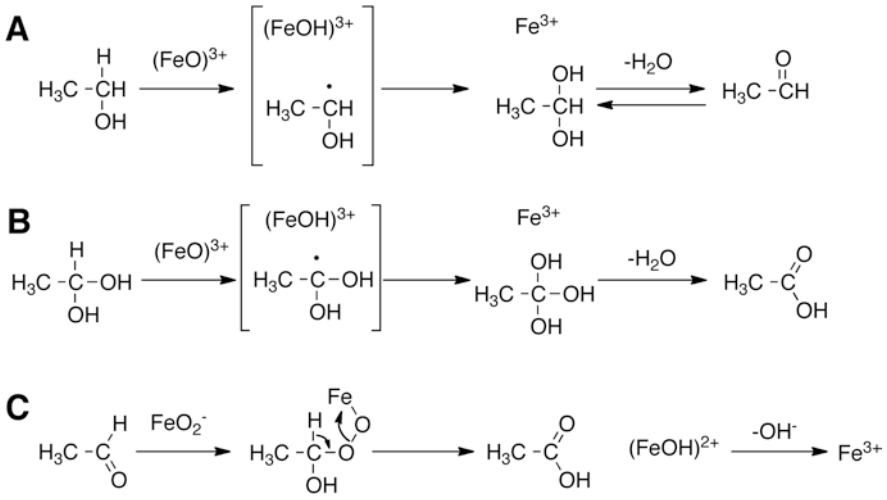

Fig. 2.2 Possible mechanisms of oxidation of ethanol to acetaldehyde and acetic acid [33]. (**a**) oxidation of ethanol to acetaldehyde. (**b**) oxidation of acetaldehyde by a perferryloxo mechanism (Compund I). *C*, oxidation of acetaldehyde by a ferric peroxide mechanism

We were interested in kinetic hydrogen isotope effects (KIEs) for ethanol oxidation because of some reports on KIEs for nitrosamine oxidation by liver microsomes [41, 49]. Work with recombinant human P450 2E1 showed, not unexpectedly, that α-deuterated ethanol showed a 5-fold KIE, expressed in the K_m but not k_{cat} (Fig. 2.3) [11]. This was shown to be the result of "burst" kinetics, i.e. a rate-determining step following product formation [11, 32]. The kinetic analysis is not novel [79], and K_m is a constant that obviously has nothing to do with K_d here (a general axiom in enzymology) [35, 56]. K_m even contains k_{cat} as a variable in this case [32]. That is, for the simplified expression

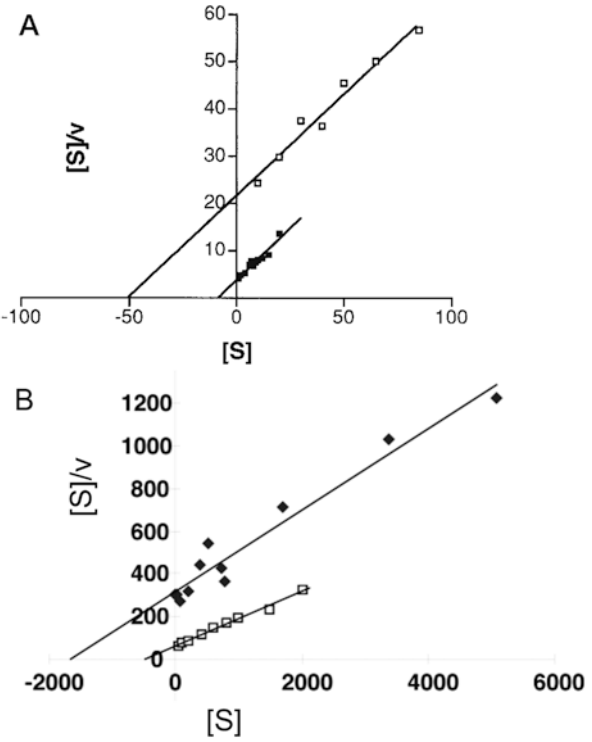

Fig. 2.3 Kinetic deuterium isotope effects with human P450 2E1 [11, 12]. The results are presented in Hanes-Wolff plots ([S]/v vs. [S]). (**a**) ethanol to acetaldehyde. CH_3CH_2OH as substrate (k_{cat}, 2.7 (± 0.2) min^{-1}, K_m 11 (± 2) mM); CH_3CD_2OH as substrate (■, k_{cat} 2.4 (± 0.1) min^{-1}, K_m 53 (± 6) mM). (**b**) acetaldehyde to acetic acid. CH_3CHO as substrate (□, k_{cat} 7.5 (± 0.5) min^{-1}, K_m 0.50 (± 0.2) mM); CH_3CDO as substrate (◆, k_{cat} 5.0 (± 0.4) min^{-1}, K_m 1.5 (± 0.3) mM). This research was originally published in Bell, L. C., and Guengerich, F. P. [11] Oxidation kinetics of ethanol by human cytochrome P450 2E1. Rate-limiting product release accounts for effects of isotopic hydrogen substitution and cytochrome b_5 on steady-state kinetics. *The Journal of Biological Chemistry* **272**, 29,643–29,651 and Bell-Parikh, L. C., and Guengerich, F. P. [12] Kinetics of cytochrome P450 2E1-catalyzed oxidation of ethanol to acetic acid via acetaldehyde. *The Journal of Biological Chemistry* **274**, 23,833–23,840.

$$E \underset{k_2}{\overset{k_1 S}{\rightleftarrows}} ES \overset{k_3}{\rightarrow} EP \overset{k_5}{\rightarrow} E + P$$

where S is the substrate and P the product. Then

$$V_{max} = \frac{k_3 k_5}{k_3 + k_5}[E]_T$$

$$K_m = \frac{k_5 (k_2 + k_3)}{k_1 (k_3 + k_5)}$$

with K_m having units of molarity. k_3 must be considerably larger than k_5, the rate of a step following product formation [44]. The original expressions for V_{max} and K_m are then reduced to

$$V_{max} \cong k_5 [E]_T$$

$$K_m = \frac{k_5 (k_2 + k_3)}{k_1 k_3} \cong \frac{k_5 k_2}{k_1 k_3} + \frac{k_5}{k_1}$$

K_m even contains k_{cat} as a variable in this case [32].

We also showed that (human) P450 2E1 can oxidize acetaldehyde to acetic acid [12], in support of reports by others with the rat enzyme [45, 75] (Figs. 2.2, 2.3b, c). This oxidation also displays burst kinetics [12] and a KIE in the K_m parameter. Pulse-chase experiments showed that the conversion of radiolabeled ethanol into acetic acid was not attenuated by the addition of unlabeled acetaldehyde. However, the results cannot be explained by a high affinity of P450 2E1 for acetaldehyde. A scheme involving a slow conformational step after the formation of acetaldehyde (and acetic acid) has been proposed [12]. Some similar results were found in the oxidation of *N*,*N*-dimethyl- and *N*,*N*-diethyl nitrosamine by P450 2E1 [17].

2.4 Source of Oxidative Stress Due to P450 2E1 in Tissues

A role for P450 2E1 in generation of reactive oxygen species (ROS) is often assumed in much of the literature in the field. Much of this view is based on early studies with liver microsomes [22, 62] and studies in cell culture [16]. The ROS field is complicated in that the vast majority of the work is *in vitro* and many of the methods of detecting ROS are not validated, e.g. see [40]. In the field, a "gold standard" is F_2-isoprostane production, which can be measured both *in vitro* and *in vivo* [38, 39].

Treatment of rats with typical P450 enzyme inducers did not produce enhanced levels of liver isoprostanes, except in the case of barbiturate-type induction [20]. In particular, treatment of rats or mice with isoniazid, an established inducer of P450

2E1 [68], did not increase isoprostane levels [20, 21]. Further, *Cyp2e1*$^{-/-}$ mice had very similar levels of liver, kidney, brain, and urinary isoprostanes [21]. Thus, P450 2E1 does not appear to increase global levels of ROS. Increased levels of ROS related to treatment of animals with ethanol are not linked only to induction of P450 2E1. Further, although the utilization of reducing equivalents (NADPH) by purified P450 2E1 is low, it is not inferior to several other (human) P450s we have worked with [74, 84].

As has been suggested in the literature [42, 83], ROS production in the presence of ethanol is probably not due to P450 2E1 induction per se but possibly to the generation (and reaction) of $CH_3CHO\cdot$ radicals.

The ROS increases following treatment of rats and mice with barbiturates have been attributed to induction of nicotinamide *N*-methyl transferase and depletion of pyridine nucleotides to support ROS destruction [21].

2.5 Roles of Mitochondrial P450s

Of the 57 human P450s (Table 2.1), seven are *bona fide* mitochondrial enzymes (11A1, 11B1, 11B2, 24A1, 27A1, 27B1, 27C1). These P450s have distinct roles in the metabolism of sterols and fat-soluble vitamins and they utilize electrons supplied by NADPH-adrenodoxin reductase (ADR) and the iron-sulfur protein adrenodoxin (Adx) [29].

The other 50 P450 proteins, when expressed, are targeted to the endoplasmic reticulum (ER). However, fractions of these P450s can, in some cases, be found in mitochondria. This was first shown in rats, where P450 2B1 could be found in mitochondria and catalyze the oxidation of aflatoxin B_1 [53]. Further studies established this phenomenon, i.e. partial localization in mitochondria and catalytic activity there [55, 71]. Studies from several groups showed that mitochondrial Adx (plus ADR) and bacterial ferredoxin (plus NADPH-ferredoxin reductase) could efficiently donate electrons to mitochondria-localized P450 1A1, 2E1, 2C8, 2B1, 2D6, 3A4, and 17A1 [5, 6, 9, 19, 59, 70], demonstrating that mitochondria-localized P450s are catalytically active. It was also shown that the N-terminal acidic domain of Adx interacted with mitochondria-localized P450 1A1 through charge-charge interaction [1]. These Adx-interacting domains are conserved in other P450 Family 1 members and also in P450s 2B1, 2B4, 2D6, 2E1, 3A4, 17A1, and 21A2 [5, 6, 9, 46, 59, 70].

One of the important factors in signaling proteins for endoplasmic reticulum (ER) localization is signal recognition particle (SRP) [69]. SRP interacts with hydrophobic residues in the N-terminal regions of proteins declined for ER localization. We reasoned that the introduction of charged residues in the N-terminal region of rat P450 2E1 might lead to decreased SRP recognition and increased mitochondrial import [7]. This hypothesis was tested, and positive results were obtained, particularly with the Mt^{++} derivative (Fig. 2.4). Mitochondria from cells

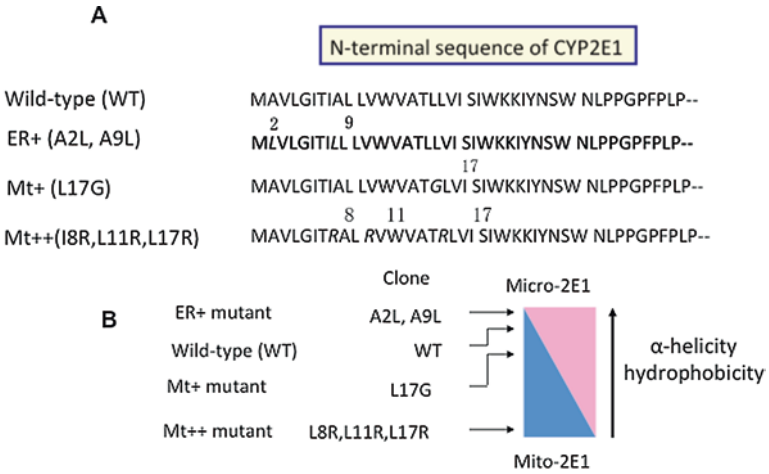

Fig. 2.4 A mutational approach for altering the bimodal targeting efficiency of rat P450 2E1 [7]. (**a**) the WOLFPSORT program was utilized to alter the SRP binding and mitochondria-targeting efficiencies of the N-terminal signal regions. (**b**) predicted targeting efficiencies of WT and mutant P450 2E1 proteins. This research was originally published in Bansal, S., Liu, C. P., Sepuri, N. B., Anandatheerthavarada, H. K., Selvaraj, V., Hoek, J., Milne, G. L., Guengerich, F. P., and Avadhani, N. G. [7] Mitochondria-targeted cytochrome P450 2E1 induces oxidative damage and augments alcohol-mediated oxidative stress. *The Journal of Biological Chemistry* **285**, 24,609–24,619.

transfected and expressing the Mt^{++} variant also showed increased mitochondrial ROS production, as judged by isoprostane measurements (Fig. 2.5). When the variants were expressed in yeast, the cell showed similar phenotypes when the yeast were grown in glucose medium regardless of the N-terminal sequence (Fig. 2.6). However, with the non-fermentable carbon source lactic acid in the media, the cells transfected with the Mt^{++} variant, localized to the mitochondria, showed selective toxicity. The phenotype was petite, indicating a loss of mitochondrial function.

Consistent with the data obtained with COS and yeast cells expressing the Mt^{++} variant of P450 Δ2E1, hepatic mitochondria from rats fed ethanol showed a time-dependent increase in F_2-isoprostane production, while hepatic microsomal membranes from these rats showed no significant increase [7]. The increased mitochondrial ROS production was accompanied by attenuated mitochondrial DNA contents, as well as cytochrome *c* oxidase and other electron transport complex activities, suggesting that mitochondrial P450 2E1 promotes alcohol-mediated mitochondrial dysfunction [1, 2, 13, 66, 70]. A recent study with livers of alcohol-fed mice suggested that mitochondrial P450 2E1 contributes significantly to the metabolic profiles of the liver tissue with several P450 2E1 substrates, including 4-nitrophenol, aniline, and styrene [34].

Studies with rat P450 2E1 [3, 67] showed that the N-terminal 160 amino acid region of the protein contained signals for targeting to both the ER and mitochondria. The positively charges residues of immediately upstream of the transmembrane

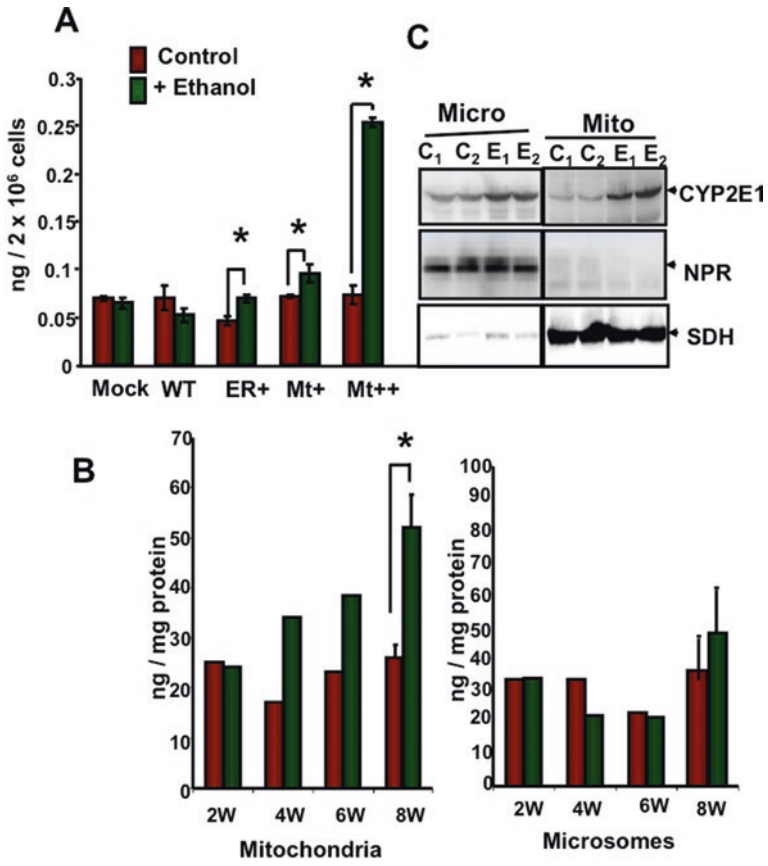

Fig. 2.5 Ethanol-induced F_2-isoprostanes in P450 2E1-expressing cells and in liver fractions from ethanol-treated rats [7]. (**a**) F_2-isoprostanes were assayed using gas chromatography-mass spectrometry. Asterisks represent significant increase in F_2-isoprostanes in ER^+, Mt+, and Mt^{++} cells after ethanol treatment ($p < 0.05$). Values represent the means ± SD of three assays. *B*, F_2-isopreostanes were measured in mitochondria and microsomes isolated from the livers of rats fed with alcohol for 2–8 weeks (W) and pair-fed controls. In each case 100 μg of protein was used. The means ± SD in the 8-week-fed rats were based on assays carried out in three rats each in control and fed groups. Asterisks represent significant difference ($p < 0.05$) from pair-fed controls. The values presented in boxes below the graph indicate the ratios of P450 (CYP) 2E1 contents between pair-fed controls and alcohol-fed rat livers. This research was originally published in Bansal, S., Liu, C. P., Sepuri, N. B., Anandatheerthavarada, H. K., Selvaraj, V., Hoek, J., Milne, G. L., Guengerich, F. P., and Avadhani, N. G. [7] Mitochondria-targeted cytochrome P450 2E1 induces oxidative damage and augments alcohol-mediated oxidative stress. *The Journal of Biological Chemistry* **285**, 24,609–24,619. © The American Society for Biochemistry and Molecular Biology

domain of the protein functioned as a cryptic mitochondria targeting signal. Furthermore protein kinase A-mediated phosphorylation at Ser-129 enhanced mitochondrial translocation of the protein through enhanced affinity for binding to cytosolic HSP70 chaperone protein—which has been implicated in presenting

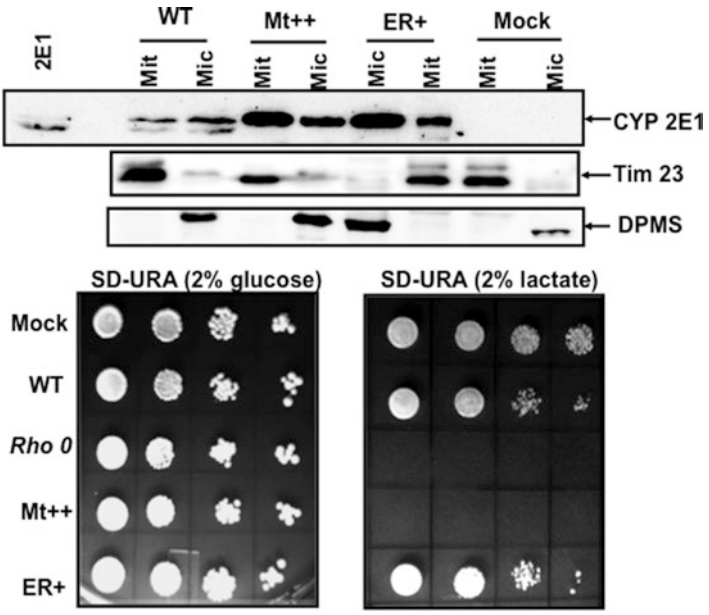

Fig. 2.6 Mitochondrial P450 2E1-induced respiratory deficiency in yeast cells [7]. (**a**) mitochondrial and microsomal CYP2E1 contents in yeast cells stably expressing WT and mutant rat P450 (CYP) 2E1 cDNA constructs. The mitochondrial and microsomal proteins (50 μg each) were analyzed using immunoblotting with anti-P450 2E1. Two identically run (parallel) blots were probed with antibody to the mitochondria-specific marker Tim23 and the microsome-specific marker dolicholphosphate mannose synthase (DPMS). (**b**), yeast cells expressing ER^+, WT, and Mt^{++} rat P450 2E1 were grown in yeast extract/peptone/dextrose medium supplemented with appropriate amino acids. Cells (~ 2.0 OD_{600} units) were pelleted and resuspended in 1 ml of sterile water. The culture was serially diluted 10 times, and 10 μl of each dilution was spotted onto plates containing 2% glucose (w/v) (left panel) and 2% lactate (w/v) (right panel), which were incubated at 30 °C for 4 days. This research was originally published in Bansal, S., Liu, C. P., Sepuri, N. B., Anandatheerthavarada, H. K., Selvaraj, V., Hoek, J., Milne, G. L., Guengerich, F. P., and Avadhani, N. G. [7] Mitochondria-targeted cytochrome P450 2E1 induces oxidative damage and augments alcohol-mediated oxidative stress. *The Journal of Biological Chemistry* **285**, 24,609–24,619.

mitochondrial passenger proteins to mitochondrial outer membrane translocators. Subsequent work [7] showed that the hydrophobicity of the N-terminal targeting domains of P450 Family 2 proteins determines their affinity for SRP binding and thus the level of ER vs. mitochondrial targeting [7]. P450 2E1—with a more hydrophobic N-terminus—was mostly targeted to the ER, and proteins with higher hydrophilicity were increasingly targeted to mitochondria.

Analysis of human liver samples from our collection showed varying degrees of mitochondrial localization, > 90% in some cases [8] (Fig. 2.7). DNA sequence analysis showed the presence of nucleotides coding for positively charged residues in the N-terminal region of P450 2E1 in these human samples. Liver samples with high mitochondrial P450 2E1 contents showed reduced cytochrome *c* oxidase

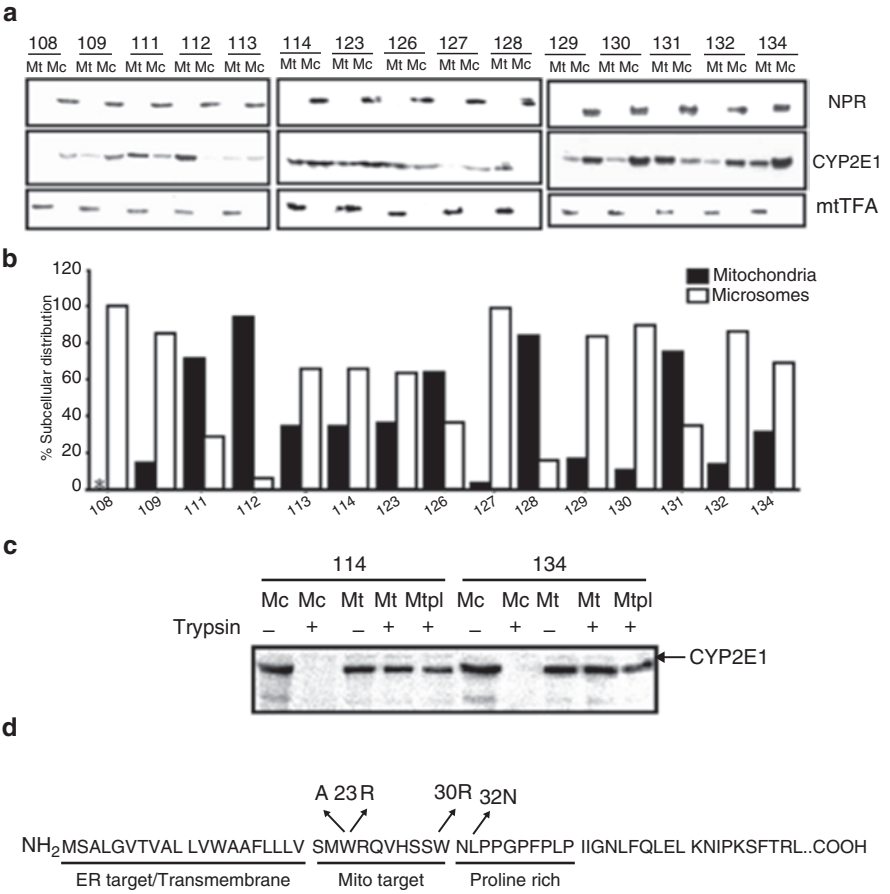

Fig. 2.7 Interindividual variations in P450 2E1 content of human liver samples [8]. **(a)** immunoblotting analysis of mitoplast (Mt) and microsomal (Mc) fractions isolated from human liver samples (50 μg of protein each) using polyclonal antibodies to human P450 (CYP) 2E1 (1:1000, v/v) and the mitochondrial marker protein mtTFA (1:3000, v/v) and a monoclonal antibody to microsomal NADPH-P450 reductase (1:1500, v/v). (**b**) densitometric analysis was performed to determine the distribution of P450 2E1 in human mitochondria and microsomes. (**c**) immunoblot analysis (with anti-human P450 2E1) of human liver mitochondrial and microsomal proteins from liver samples (HL, 'human liver') HL114 and HL134, subjected to limited trypsin digestion (150 μg/mg protein, 20 min on ice). **(d)** N-terminal amino acid sequence of human P450 2E1 protein indicating the ER targeting domain, mitochondrial targeting domain, and proline-rich domain. Variations within the putative signal sequence region are shown by the arrows. This research was originally published in Bansal, S., Anandatheerthavarada, H. K., Prabu, G. K., Milne, G. L., Martin, M. V., Guengerich, F. P., and Avadhani, N. G. [8] Human cytochrome P450 2E1 mutations that alter mitochondrial targeting efficiency and susceptibility to ethanol-induced toxicity in cellular models. *The Journal of Biological Chemistry* **288**, 12,627–12,644. © The American Society for Biochemistry and Molecular Biology

activity and markedly depleted cytochrome *c* oxidase I, IVi1, and Vb subunits [8]. Consisted with observations with the rat P450 2E1, the N-terminal 160 amino acids of human P450 2E1 were sufficient to direct the organelle localization of P450s [8]. Accordingly, this region could be fused to a reporter protein (dihydrofolate reductase, DHFR) to monitor membrane localization. Sequence analysis of the N-terminal coding regions cDNAs from human liver samples showed W23R and W30R substitutions and, as with rat P450 2E1 [7], the N-terminal sequence variants with the more basic residue substitutions (i.e., W23R/W30R) showed higher mitochondrial translocation and more mitochondrial ROS production (Fig. 2.8). In other assays, mitochondria from HepG2 cells transfected with the mitochondria-directed variants showed increased uncoupled respiratory rates and partial mitochondrial DNA depletion (Fig. 2.9). Thus, although the direct role of P450 2E1 in ROS production at the whole organ level remains suspect, the mitochondria-targeted P450 2E1 appears to augment alcohol toxicity by inducing mitochondrial respiratory dysfunction.

2.6 Future Questions

Collectively, the studies with mitochondria show a selective response of P450 2E1 localized there, in terms of ROS production. The mitochondrial P450 2E1 showed a similar catalytic activity in *N*,*N*-dimethylnitrosamine oxidation (as the microsomal P450 2E1) [7]. The results, taken collectively, argue for a selective role of mitochondrial P450 2E1 in ethanol toxicity. Exactly how this relates to ethanol-linked cancer is not clear. P450 2E1 may be generating more mitochondrial DNA adducts, and there is evidence for a higher level of DNA adducts in mitochondria with some carcinogens [4, 54, 78]. What is not clear is how genetic mutations in the mitochondrial DNA would yield tumors, in the context of current theories regarding roles of mutations in oncogenes and tumor suppressor genes.

Another basic question is how Adx interacts with mitochondrial P450 2E1 to deliver electrons. Several other human P450s have now been found to have significant fractions localized in mitochondria (i.e., 1B1, 2C8, 2D6) [5, 6, 9, 29, 70] and this is a similar question in these cases. One possibility is that electron transfer to the P450 with Adx occurs through a different domain, or with altered efficiency as with the normal redox partner NADPH-P450 reductase (an ER protein) and may determine the relative activities. Additionally, the mitochondria localized P450s 1A1, 2B1, and 2E1 show significant changes in their α-helical and β-sheet contents, suggesting altered folding. Many of these questions remain to be addressed in a more detailed manner.

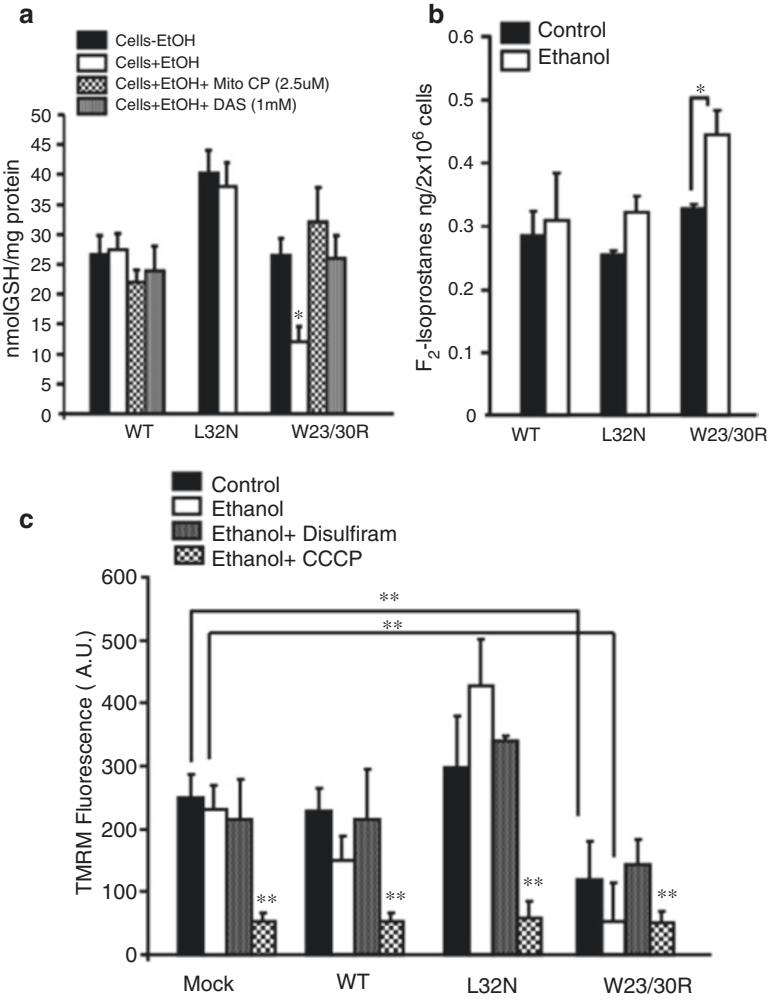

Fig. 2.8 Effects of ethanol on cellular toxicity in cells expressing WT and mutant human P450 2E1 constructs [8] (**a**) cellular GSH levels (nmol/mg protein). (**b**) ethanol-induced F_2-isoprostanes in human P450 2E1-expressing cells, assayed using gas chromatography-mass spectrometry (expressed as ng/2 × 10^6 total cells). (**c**) mitochondrial membrane potential ($\Delta\Psi_m$) measured spectrophotometrically in stable cells using a fluorescent dye, tetramethylrhodamine methyl ester (TMRM). Briefly, cells were incubated with and without ethanol and also treated with disulfiram (25 μM) overnight. Cells were washed and loaded with 150 nM tetramethylrhodamine methyl ester in an assay involving a Chameleon microplate reader (excitation wavelength 535 nm, emission wavelength 590 nm). For a control, 10 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was used in each series. *, p <0.05; **, p <0.001. Values represent means ± SE from three independent measurements. A.U., arbitrary units. This research was originally published in Bansal, S., Anandatheerthavarada, H. K., Prabu, G. K., Milne, G. L., Martin, M. V., Guengerich, F. P., and Avadhani, N. G. [8] Human cytochrome P450 2E1 mutations that alter mitochondrial targeting efficiency and susceptibility to ethanol-induced toxicity in cellular models. *The Journal of Biological Chemistry* **288**, 12,627–12,644. © The American Society for Biochemistry and Molecular Biology

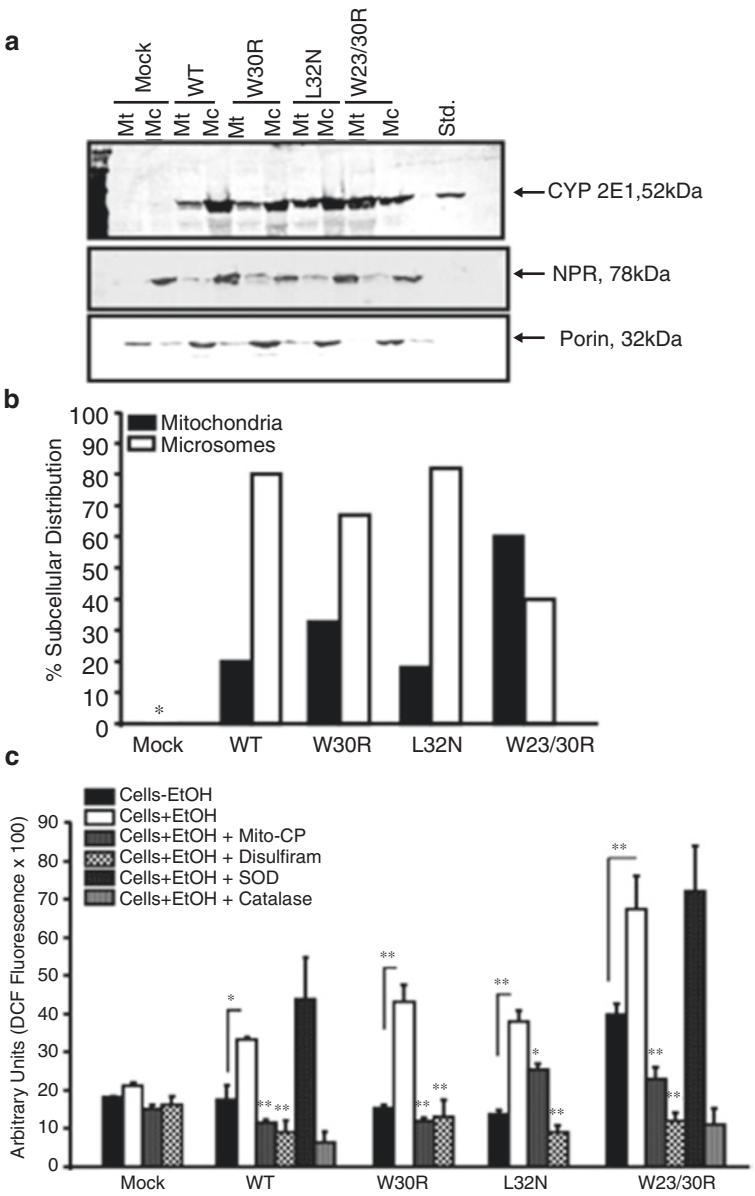

Fig. 2.9 Subcellular distribution and ethanol-mediated ROS in HepG2 cells stably expressing WT or mutant human P450 2E1. [8] (**a**) mitochondria and microsomes from HepG2 cells stably expressing human P450 2E1 cDNAs. Mitochondrial and microsomal fractions (50 μg of protein) were subjected to immunoblotting analysis (anti- human P450 2E1). The blot was also co-developed with anti-NADPH-P450 reductase (a microsomal marker) and porin (a mitochondrial marker) to assess relative cross-contamination. (**b**) percentage subcellular distribution was calculated based on band intensity. (**c**) ROS levels in whole cells grown with or without ethanol (300 mM) were estimated using the dye dichlorofluorescin (DCF) as the substrate.

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Fig. 2.9 (continued) Cells were treated with the mitochondria-targeted antioxidant Mito-CP (2.5 μM)and the P450 2E1 inhibitor disulfiram (25 μM) (cell-permeable superoxide dismutase and catalase were used as controls). Values represent means ± SD (error bars) from three independent experiments. *, $p < 0.05$; **, $p < 0.001$. This research was originally published in Bansal, S., Anandatheerthavarada, H. K., Prabu, G. K., Milne, G. L., Martin, M. V., Guengerich, F. P., and Avadhani, N. G. [8] Human cytochrome P450 2E1 mutations that alter mitochondrial targeting efficiency and susceptibility to ethanol-induced toxicity in cellular models. *The Journal of Biological Chemistry* **288**, 12,627–12,644. © the American Society for Biochemistry and Molecular Biology

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Chapter 3
Glutathione and Transsulfuration in Alcohol-Associated Tissue Injury and Carcinogenesis

Ying Chen, Ming Han, Akiko Matsumoto, Yewei Wang, David C. Thompson, and Vasilis Vasiliou

Abstract Glutathione (GSH) is the most abundant non-protein thiol, attaining cellular concentrations in the millimolar range. GSH functions to protect cells against endogenous and exogenous electrophiles. In addition, GSH serves as a cofactor for the GSH peroxidase family of enzymes which metabolize H_2O_2 as well as lipid peroxides. Through the action of glutathione S-transferase family of enzymes, GSH is conjugated to a variety of electrophilic endogenous compounds and exogenous chemicals, and thereby facilitates their efficient and safe elimination. Through the transsulfuration pathway, GSH biosynthesis is metabolically linked with cellular methylation, which is pivotal for epigenetic gene regulation. Accumulating evidence suggests that the underlying mechanisms of alcohol-associated tissue injury and carcinogenesis involve: (i) generation of the electrophilic metabolite acetaldehyde, (ii) induction of CYP2E1 leading to the formation of reactive oxygen species and pro-carcinogen activation, and (iii) nutritional deficiencies, such as methyl groups, resulting in enhanced susceptibility to cancer development. In this context, clinical and experimental investigations suggest an intimate involvement of GSH and related enzymes in the development of alcohol-induced pathological conditions.

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The aim of this review is to provide an overview of the GSH biosynthesis, cellular transsulfuration/transmethylation pathways, and their implications in the pathogenesis and treatment of alcohol-related disease and cancer.

Keywords Alcoholic · Cancer · Oxidative stress · Glutathione · Transsulfuration · Methylation

3.1 Introduction

Glutathione (GSH) is a ubiquitous tripeptide composed of glutamate, cysteine and glycine. It presents as the most prevalent non-protein thiol in mammalian cells. Extensive research has revealed numerous and diverse cellular functions of GSH [1]. It detoxifies xenobiotics and endogenous metabolites through non-enzymatic or enzymatic mechanisms. It functions as a major antioxidant to protect cells against oxidative damage caused by reactive oxygen species (ROS). As such, it is essential in maintaining the intracellular redox balance and the thiol moieties of proteins. Through such processes, GSH can modulate protein function *via* redox post-translational modification. It also plays a role in the regulation of nitric oxide homeostasis. Through the transsulfuration pathway, GSH participates in cellular shuttling of other sulfur amino acids [2]. Given the diversity and importance of these functions of GSH, it should come as no surprise that alterations in GSH levels have been found to be associated with numerous human pathological conditions, including cancer, liver disease, cardiovascular disease, neurological disorders, diabetes, and other disease conditions [3].

Oxidative stress occurs when ROS are produced at levels exceeding those capable of being sequestered by normal cellular antioxidant processes. Chronic ethanol consumption induces oxidative stress in organs *via* cellular pathways that promote the overproduction of reactive molecules (including ROS and electrophilic products, such as acetaldehyde and lipid peroxidation-derived products) and/or the diminution of antioxidant defenses, such as GSH [4]. Studies in human subjects and animal models have implicated an important mechanistic role for disrupted GSH homeostasis in the pathogenesis of alcohol-related non-cancerous diseases, particularly alcoholic liver disease [5]. The involvement of changes in the GSH redox homeostasis in alcohol-associated cancers, however, appears more complex and remains to be elucidated. This review focuses on the links between GSH, the transsulfuration pathway, and alcohol-induced tissue injury, and their involvement in the development and therapy of alcohol-related cancers.

3.2 GSH Biosynthesis, Metabolism and Function

GSH is synthesized by two successive enzymatic reactions (Fig. 3.1) [6]. The first reaction, catalyzed by glutamate-cysteine ligase (GCL), couples glutamate and cysteine to form γ-glutamylcysteine (γ-GC). The second reaction couples γ-GC with glycine and is catalyzed by GSH synthase (GSS). Each of these enzymatic reactions consumes one molecule of ATP per catalytic cycle. The formation of γ-GC by GCL is considered the rate-limiting enzymatic step in GSH biosynthesis. For this reason, GCL rather than GSS has been the principal target of drugs designed to inhibit GSH biosynthesis [7] and to generate animal models with GSH deficiency [8]. GCL of higher eukaryotic organisms, in its most catalytically efficient form, is a heterodimer composed of a catalytic (GCLC) and a modifier (GCLM) subunit, each of which is encoded by separate genes. As its name implies, GCLC possesses all of the catalytic activity of GCL, and GCLM serves to optimize the kinetic properties of GCLC [9]. Both the GCLC and GCLM genes are up-regulated by electrophiles or agents that cause oxidant stress [10] *via* transcriptional mechanisms reminiscent of phase II drug metabolizing-enzyme genes. While *GCLC* and *GCLM* genes are commonly found up-regulated together, cell type-specific differential expression of

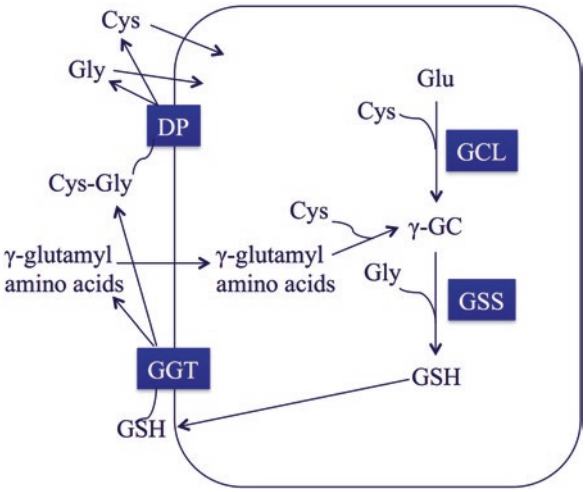

Fig. 3.1 Scheme of γ–glutamyl cycle for glutathione (GSH) biosynthesis and catabolism. GSH is synthesized by two successive enzymatic reactions. Glutamate-cysteine ligase (GCL) couples glutamate (Glu) and cysteine (Cys) to form γ-glutamylcysteine (γ-GC), which is the rate-limiting step in GSH synthesis. GSH synthase (GSS) then couples γ-GC with glycine to form GSH. GSH can be transported out of the cell where it is catabolized by γ-glutamyl transferase (GGT). GGT cleaves the γ-glutamyl amide bond between Glu and Cys releasing cysteinylglycine (Cys-Gly) and γ–glutamyl amino acids. Cys-Gly can be further cleaved by an extracellular dipeptidase (DP), producing free Cys and Gly for reuse by the cell. γ–glutamyl amino acids can be taken up by the cell to form γ-GC, essentially bypassing the need for catalysis by GCL

GCLC and GCLM transcripts suggest independent regulation of these subunits [8]. Current evidence indicates that most, if not all, of the GSH biosynthetic activity resides in the cytoplasm [11]. The GSH, thus produced, is further distributed into intracellular organelles including the mitochondria, endoplasmic reticulum (ER) and nuclei [12].

Due to the presence of a unique γ-glutamyl amide bond between the γ-carbon of the glutamate side chain and the amino group of cysteine, GSH cannot be broken down by peptidases inside the cell. Rather, GSH must be transported through the plasma membrane and out of the cell, where it is metabolized by γ-glutamyl transferases [GGTs] [6]. These enzymes catalyze the ATP-dependent cleavage of the γ-glutamyl amide bond between glutamate and cysteine, and generates cysteinylglycine that can be further cleaved by an extracellular dipeptidase (DP). This reaction produces free cysteine and glycine, which can then be used by cells. These reactions for synthesis and degradation of GSH form a metabolic pathway known as the γ-glutamyl cycle [13] (Fig. 3.1). By way of this cycle, GSH participates in amino acid transport for cellular re-synthesis of GSH and other proteins. In addition, it represents a salvage pathway by which GSH can be produced independently of GCL [14].

GSH is the most abundant cellular thiol, attaining concentrations from 1 to 10 mM depending on the cell type [11, 15, 16]. The oxidized form of GSH is glutathione disulfide (GSSG). The cellular GSH/GSSG ratio has been used as an index of cellular redox status. Under normal circumstances, this ratio exceeds 10:1; a decrease in GSH/GSSG ratio is commonly associated with increased cellular oxidative stress [17]. GSH serves to protect cells against toxicity arising from exposure to excessive amounts of endogenous and exogenous electrophiles [7]. It scavenges hydroxyl radical and superoxide directly, and serves as a cofactor for the glutathione peroxidase (GPX) enzymes in metabolizing H_2O_2, as well as lipid peroxides [18]. Through the action of the glutathione S-transferase (GST) family of enzymes, GSH may be conjugated to a variety of electrophilic endogenous compounds and exogenous chemicals, and thereby facilitates their efficient and safe elimination [19]. Together, GSH and GSSG function as an important cellular redox buffering system that has been suggested to be involved in determining cell fate decisions, such as proliferation and apoptosis [20].

In subcellular compartments, GSH plays a pivotal role in the normal functioning of mitochondria, where oxygen consumption and generation of ROS occurs. GSH in the nucleus maintains the redox status of critical protein sulfhydryl groups that are necessary for expression, transcription activity, and DNA repair [21]. In contrast to other organelles, GSH in the endoplasmic reticulum exists more in the oxidized state (GSSG), which is believed to be necessary for providing the appropriate environment for assembly and secretory pathways for proteins [22].

3.3 The Transsulfuration Pathway

Transsulfuration is a biochemical pathway that connects glutathione biosynthesis to the metabolism of sulfur-containing amino acids, *viz*., methionine and cysteine (Fig. 3.2) [23]. In the methionine cycle, methionine forms S-adenosylmethionine (SAM) in a reaction catalyzed by methionine adenosyltransferase (MAT). SAM is converted to S-adenosylhomocysteine (SAH) by the actions of methyltransferases (MTs), which transfer the methyl group to accepting molecules. Homocysteine is then derived from SAH *via* a reversible reaction catalyzed by SAH hydrolase (SAHH). Methionine can be regenerated from homocysteine by one of two methylation pathways. In the first, methionine synthase (MS) catalyzes the transfer of a

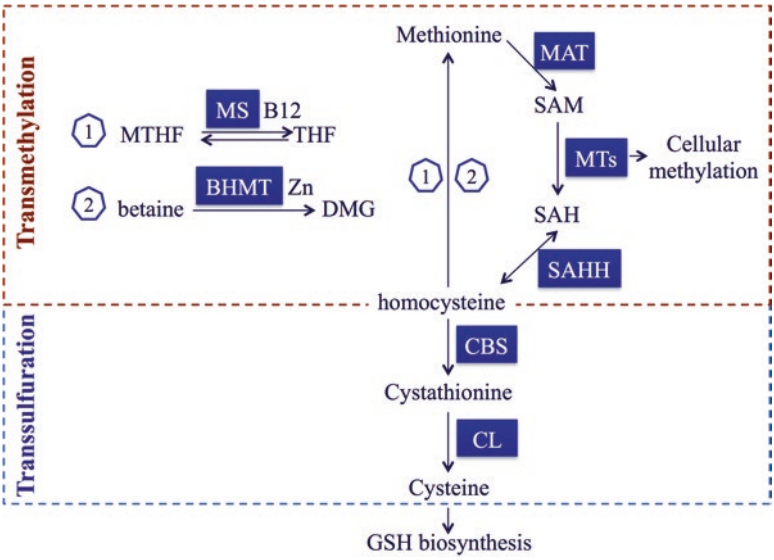

Fig. 3.2 Major enzymes and intermediates in cellular transmethylation-transsulfuration pathways. In the liver, the transsulfuration pathway connects transmethylation cycle (methionine cycle) to glutathione (GSH) biosynthesis. Methionine forms S-adenosylmethionine (SAM), the major biological methyl donor, by the action of methionine adenosyltransferase (MAT). SAM is then converted to S-adenosylhomocysteine (SAH) by the actions of various methyltransferases (MTs). These MTs transfer the methyl group to accepting molecules (e.g., DNA, RNA and proteins) undergoing methylation. Homocysteine is derived from the hydrolysis of SAH by the action of SAH hydrolase (SAHH). In the methionine cycle, methionine can be regenerated from homocysteine by one of two remethylation pathways. In one pathway (1), methionine synthase (MS) catalyzes the transfer of a methyl group from N^5-methyltetrahydrofolate (MTHF) to homocysteine creating methionine and tetrahydrofolate (THF); this reaction requires vitamin B12 (B12) as a cofactor. In the other pathway (2), betaine is the source of the methyl group transferred to homocysteine, which is catalyzed by a zinc (Zn)-dependent enzyme, betaine homocysteine methyltransferase (BHMT). The transsulfuration pathway starts with homocysteine being irreversibly converted to cystathionine by the enzyme cystathionine-β-synthase (CBS). Cystathionine is further converted to cysteine by cystathionine-γ-lyase (CL). Cysteine can then feed GSH biosynthesis

methyl group from N^5-methyltetrahydrofolate (MTHF) to homocysteine creating methionine and tetrahydrofolate (THF); this reaction requires vitamin B12 as a cofactor. In the second pathway, betaine serves as the source of the methyl group transferred to homocysteine, a reaction catalyzed by the zinc-dependent enzyme betaine homocysteine methyltransferase (BHMT). The transsulfuration pathway involves homocysteine being irreversibly converted to cystathionine by the enzyme cystathionine-β-synthase (CBS). Cystathionine is converted to cysteine by cystathionine-γ-lyase (CL). The resulting cysteine can then be used for GSH biosynthesis. In the liver, approximately 50% of the cysteine used for GSH synthesis is derived from the transsulfuration pathway from methionine [24, 25].

The functional importance of these metabolic pathways is underscored by their essentiality for cellular methylation and for the maintenance of cellular redox homeostasis. The intermediate, SAM, serves as a primary methyl donor participating in epigenetic gene regulation, protein stability, and phospholipid and neurotransmitter production [26]. Through the transsulfuration pathway, SAM has been shown to increase GSH, inhibit lipid peroxidation, and protect against oxidative stress associated with ischemia-reperfusion injury in brain tissues [27]. Deficiencies in enzymes of the transsulfuration pathway may lead to ROS generation, homocysteine accumulation and macrophage synthesis of proinflammatory molecules, and thereby contribute to human pathologies like atherosclerosis and tumor development [23]. Homocysteine accumulation induces fibrin deposition, oxidant stress, cytokine release and inflammation, promoting atherosclerosis [28].

3.4 Glutathione and Transsulfuration in Alcohol-Related Non-Cancerous Diseases

Alcohol consumption can cause a variety of health issues. Heavy drinking is associated with numerous non-cancerous health conditions, including liver disease, cardiovascular disease, disorders of the digestive tract, pulmonary disease, and neurobehavioral disorders. Oxidative stress appears to be intimately involved in the initiation and progression of these diseases [4]. Alcohol consumption induces oxidative stress through a variety of cellular changes; an important one involves compromised cellular antioxidant defense mechanisms including alterations in GSH [4]. GSH levels and/or its redox status (e.g., GSH/GSSG ratio) in the plasma and tissues from ethanol-fed animals and chronic alcoholics have been investigated in numerous studies. In rodents, chronic ethanol consumption caused decreases in heart cytosolic and mitochondrial GSH levels and concomitant increases in cytosolic and mitochondrial levels of lipid peroxidation and protein carbonyls; such compromised oxidant buffering capacity has been proposed to contribute to the pathogenesis of alcoholic cardiomyopathy [29]. The impact of chronic alcoholism on systemic and pulmonary GSH redox status was investigated

in a cohort comprising healthy alcohol-dependent subjects and control subjects [30]. Chronic alcoholics showed dramatic oxidant stress in the alveolar space manifesting as decreased GSH, increased GSSG, and a corresponding oxidative shift in the redox potential of GSH/GSSG. Systemic oxidative stress was observed in alcoholics who also smoked. Interestingly, alcohol-induced chronic oxidant stress in the alveolar space may sensitize alcohol abusers to acute respiratory distress syndrome [31].

The liver is a major organ subject to ethanol-induced toxicity. There is a wealth of data from studies in human and experimental animals documenting ethanol-induced changes in hepatic GSH homeostasis, including GSH/GSSG and GSH-related antioxidant enzymes [32–35]. Collectively, these studies suggest that depletion of hepatic GSH, particularly mitochondrial GSH, is one of the early changes associated with chronic ethanol consumption [36]. Importantly, plasma GSH concentrations are inversely correlated with the degree of liver damage and hepatic lipid peroxidation [32–34]. Prolonged ethanol consumption has been reported to inhibit multiple steps in methionine metabolism and transsulfuration pathways in the liver, resulting in increased homocysteine and SAH levels, and a lowered heptaic SAM/SAH ratio [37, 38]. Enzymes affected directly or indirectly by ethanol include MAT, BHMT and various methytransferases [37, 39]. The detrimental consequences of these changes include, but are not limited to, dysregulation of gene expression (due to altered DNA methylation), homocysteine-promoted inflammation, and inhibition of GSH biosynthesis [37, 39]. Importantly, serum levels of intermediates of the transsulfuration pathway (such as cystathionine) have been proposed as diagnostic markers for the severity of alcoholic liver disease (ALD) [40].

In a recent study, we utilized a transgenic mouse model to elucidate the role of GSH redox homeostasis in the hepatic response to chronic ethanol consumption [41]. Global disruption of the *Gclm* gene (GCLM knockout) results in mice that have greatly reduced (10–40% normal) tissue GSH and lower plasma GSH/GSSG [42]. In the liver, 85% depletion of GSH results in an oxidative shift of hepatic GSH redox potential by 65 mV, 60% decrease in mitochondrial GSH pool and yet mitochondrial functioning remains intact [43]. Thus, GCLM knockout (KO) mice represent a model of chronic hepatic and systemic oxidative stress. Following chronic ethanol consumption, these mice are unexpectedly protected from ethanol-induced steatosis and liver damage [41]. At the molecular level, this protective phenotype appears to involve following beneficial cellular adaptions: (i) suppression of lipogenic genes and induction of genes involved in fatty acid oxidation, (ii) induction of the nuclear-factor-erythroid 2–related-factor 2 (NRF2) antioxidant response, and (iii) activation of the AMP-activated protein kinase (AMPK) metabolic signaling pathway [41]. Our study showed unconventional beneficial cellular consequences associated with GSH deficiency, implying that hepatic GSH homeostasis may function to modulate metabolic and stress responses to ethanol consumption.

3.5 Alcohol-Mediated Carcinogenesis

Ethanol and its direct metabolite acetaldehyde have been identified as human carcinogens by the International Agency for Research on Cancer (IARC). Available epidemiological studies have established that alcohol consumption is strongly associated with an increased risk for cancers of stomach, oropharynx, larynx, oesophagus, head and neck, liver, pancreas, female breast, colorectum, and gallbladder [44–46]. In this context, alcohol is estimated to have contributed to 3.2–3.7% and 5.8% of cancer deaths worldwide and in the United States, respectively [47].

Drinking patterns play an important role in influencing the relationship between alcohol and cancer risk. An increased risk of breast cancer is associated with chronic alcohol consumption and it occurs in a dose-dependent manner [48, 49]. Consumption of 10 g alcohol each day raises the risk by 8% for post-menopausal breast cancer, 9% for pre-menopausal breast cancer, and 10% for overall breast cancer [49]; risk increases by ≈7% for every additional 10 g alcohol consumed each day [49]. A dose-dependent association also exists between lifetime alcohol intake and the risk of upper-aero digestive tract (UADT) cancer (e.g., of the oral cavity, pharynx, larynx or oesophagus) (multivariable-adjusted relative risk was 2.67 for an intake of ≥40 g/day, and 1.16 for a 10 g/day increment in intake) [50]. For the lower digestive tract, longer duration and higher amount of alcohol consumption were associated with increased colorectal cancer risk (relative risk was 2.24 for ≥30 g/day) [51–54]. While the main causal factor of hepatocellular carcinoma (HCC) is chronic infection with hepatitis B (HBV) and C (HCV) viruses, alcohol intake represents an independent risk factor for HCC [55, 56]. Chronic ethanol consumption can cause a spectrum of ALDs, which clinically can manifest as steatosis, steatohepatitis, fibrosis, and cirrhosis [57]. Only ≈1~2% of cirrhotic patients develop HCC [58]. Daily alcohol ingestion exceeding 20.44 g was associated with higher risks of both liver cancer occurring and liver disease mortality [59]. The dose-response relationship between alcohol consumption and liver cancer was apparent with relative risks of 1.54 for 50 g/day, 2.14 for 75 g/day, 3.21 for 100 g/day, and 5.20 for 125 g/day [60]. It should be noted that a J-shaped dose-response relationship between alcohol consumption and all-cause or all-cancer mortality was observed, implicating a possible beneficial effect of light drinking [61–63].

The exact molecular mechanisms causing alcohol-associated carcinogenesis are not well understood. Several have been proposed and are reviewed in depth elsewhere [58, 64, 65]. Alcohol is thought to exert carcinogenic effects at many levels, including acetaldehyde formation, induction of CYP2E1, oxidative stress, epigenetic alterations due to a reduced capacity for methyl moiety transfer, and modulation of cellular growth [58]. Alcohol is metabolized primarily *via* oxidation to acetaldehyde through the actions of alcohol dehydrogenases (ADHs) and, to a lesser extent, CYP2E1 and catalase. Acetaldehyde is then oxidatively detoxified to acetate by the aldehyde dehydrogenase enzymes (ALDHs) [66]. Acetaldehyde is a

highly reactive molecule capable of adducting DNA and proteins [67, 68]. Mitochondrial ALDH2 is the primary ALDH enzyme responsible for the elimination of acetaldehyde [69]. Human subjects carrying a defective allele of the *ALDH2* gene (*ALDH2*2* allele) have a greatly reduced capacity (10–45% normal in heterozygotes and 1–5% normal in homozygotes) to metabolize acetaldehyde [70]. Epidemiological studies have revealed these individuals to be highly susceptible to the development of gastrointestinal cancers following excessive alcohol consumption [71]. Following chronic ethanol consumption, acetaldehyde-DNA adducts are elevated to a greater extent in the liver and stomach of *Aldh2* KO mice than in wild-type mice [72, 73]. Studies in humans and experimental animals have established that acetaldehyde-DNA adduct formation is an initial step in ethanol-induced carcinogenesis [74]. Alcohol induction of CYP2E1 serves as an important molecular pathway by which ethanol can promote carcinogenicity [65]. Specifically, CYP2E1 activation may bioactivate other procarcinogens and is an important cellular source of ROS formation, including superoxide anion, hydrogen peroxide and the lipid peroxidation by-products malondialdehyde and 4-hydroxynonenal (4-HNE) [65]. 4-HNE can form highly mutagenic DNA-adducts; such adducts are more frequently observed in advanced stages of ALD [75, 76]. In addition to CYP2E1 activation, ethanol-induced oxidative stress can arise from dysfunctional mitochondrial respiration, iron overload, inflammation and/or compromised antioxidant defenses [77]. The epigenetic aspect of alcohol-induced carcinogenesis has been the subject of extensive studies in recent years and is covered in comprehensive reviews elsewhere [78, 79]. Accumulating lines of evidence suggest that ethanol consumption causes aberrant patterns of DNA methylation and thereby altered gene expression by inhibiting key enzymes involved in SAM bioavailability and DNA methyltransferases [79]. Finally, chronic ethanol consumption lowers hepatic concentrations of vitamin A and retinoic acid, which are critical modulators of cellular growth and differentiation. Importantly, an apparent inverse relationship appears to exist between serum concentrations of vitamin A and later development of HCC in humans [80, 81].

3.6 GSH and Transsulfuration in Cancer Biology and Alcohol-Related Cancers

GSH appears to play a paradoxical role in cancer biology. Firstly, oxidative stress due to production of ROS and/or electrophilic metabolites is an important mutagenic mechanism for numerous physical (e.g., ultraviolet light exposure) and chemical (e.g., alcohol) carcinogens [82–84]. GSH scavenges DNA-damaging free radicals directly or *via* enzymatic reactions (e.g., GPXs and GSTs), and in doing so, it may contribute to the prevention of tumor initiation [85, 86]. Secondly, some oncogenes (e.g., AP-1) and tumor suppressors (e.g., P53) are transcription factors that play key roles in controlling cell proliferation and death in response to genomic

stress. The DNA-binding activity of these proteins requires the maintenance of some crucial cysteine residues in a reduced form [87, 88]. By acting as a major homeostatic redox buffer in subcellular compartments, GSH-GSSG couple may modulate the activities of tumor suppressors or oncoproteins, thereby contributing to tumor promotion [89]. Thirdly, many highly metastatic cancer cells attain high intracellular levels of GSH; such a situation is typically associated with higher expressions of γ-glutamyl cycle enzymes, such as GCL and GGT [90–92]. These biochemical features are believed to function at multiple levels to promote the growth advantage and metastasis of neoplastic cells, such as: (a) the γ-glutamyl cycle supplies the fast turnover of cysteine and other amino acids for protein synthesis, (b) high GSH helps to maintain mitochondrial functional integrity to meet the high metabolic demands of the neoplastic cells, and (c) GSH combats harmful ROS or reactive nitrogen species (RNS) released by vascular endothelial cells in response to cancer cell contact in the process of metastatic invasion. Lastly, resistance of cancer cells to radiation and chemotherapy appears to correlate directly to their GSH levels. This is often accompanied by over-expression of multidrug resistance-associated proteins (MRPs) and GST enzymes [93–96]. Several mechanisms have been proposed for the role of GSH in regulating drug resistance of cancer cells: (a) GSH may directly protect against oxidative cytotoxicities elicited by anti-cancer treatments, (b) MRPs are a family of ATP-binding cassette membrane transporters that mediate the efflux of GSH and GSH-conjugates; GSH may facilitate the export of anti-cancer drugs through the actions of MRP proteins, and (c) GSTs are phase II detoxification enzymes that catalyze GSH conjugation with different chemotherapeutic compounds for their safe elimination; GSH may promote GST-mediated metabolic elimination of anti-cancer drugs by serving as its cofactor. The latter two mechanisms may act independently or cooperatively to diminish the therapeutic effects of anticancer drugs in cancer cells expressing high levels of GSH. Taken together, GSH seems to have bidirectional functions such that it can protect against neoplastic transformation in non-tumor cells while also being able to promote metastasis and chemoresistance of neoplastic cells.

Deficiencies in the transsulfuration pathway have been documented to occur in cancer cells and cancerous tissues [23]. Genetic polymorphisms in the *CBS* gene (which encodes the enzyme converting homocysteine to cystathionine) have been associated with increased risks for breast, gastrointestinal and lung cancers [97–99]. The importance of the transsulfuration pathway in cancer biology attributes partially to its metabolic link to the metabolism of cysteine and GSH [23]. The transsulfuration pathway also connects to the methionine cycle through homocysteine. A blockade of this pathway results in homocysteine accumulation as well as altered cellular transmethylation [23], both of which have been implicated in tumor initiation and progression. Homocysteine is a pro-inflammatory intermediate that causes ROS production, cytokine release, and altered expression of adhesion molecules [100]. Elevated levels of homocysteine induce chronic inflammation and are an established risk factor for coronary heart disease [28]. Many tumor cells both require high methionine for growth and export large amounts of homocysteine [101]. The elevated production of homocysteine by methionine-dependent cancer

cells is proposed to act as an adaptive mechanism that promotes a cancer microenvironment for cancer cell survival, colonization and vascular invasion [102, 103]. In the methionine cycle, accumulated homocysteine can be converted to SAH that, as a potent inhibitor of cellular methylation, can lead to SAM deficiency [104]. In agreement with this notion, aberrant DNA methylation is often observed in hyperhomocysteinemia-associated pathologies (including cancer) and is considered an important causal factor of the disease condition [104].

In the context of alcohol-related cancer, ethanol-induced depletion of the cellular GSH pool and inhibition of transsulfuration/transmethylation pathways are of particular importance for the development of alcoholic HCC. Clinically, low levels of hepatocellular GSH and SAM and a low SAM/SAH ratio are commonly observed in chronic alcoholics with advanced stage ALD and they correlate with the severity of liver damage [32, 37, 38]. The significance of reduced SAM production in the development of HCC is supported by several findings: SAM feeding blocked the transformation of pre-neoplastic lesions into HCCs [105], SAM administration inhibited the expressions of selected proto-oncogenes [106], SAM decreased the survival of liver tumor cells *in vitro* in a dose-dependent manner [107], and SAM treatment prevented liver tumor formation in a xenograft model [108]. The proposed mechanisms underlying a protective role of SAM against alcoholic HCC, including providing precursors for GSH biosynthesis and supplying methyl groups for balanced DNA methylation, are presented and discussed comprehensively in other articles [109, 110].

Along with abstinence from alcohol and anti-inflammatory treatment, nutrient (e.g., SAM) and antioxidants (e.g., GSH) supplementation represents an important element for preventive and therapeutic management of ALD including cancer [111, 112]. The use of GSH precursors (e.g., *N*-acetylcysteine) [113, 114], intermediates of transmethylation pathway (e.g., SAM, folate and betaine) [110] and compounds possessing antioxidant properties (e.g. vitamin E and plant extracts) [115, 116] have been investigated in experimental animal models and pilot human studies targeting at advanced ALD. These studies have provided inconsistent results in that human studies largely showed no beneficial effects in improving clinical markers of chronic liver damage or preventing degeneration into hepatocellular carcinoma [117, 118]. However, the lack of therapeutic efficacy of these compounds may be related to their complex pharmacokinetics in ALD patients. Nevertheless, it has been proposed that long-term use of antioxidants (including SAM) may assume a greater role for the treatment of ALD patients who are in the process of achieving sobriety and at risk for progression to cirrhosis and HCC.

3.7 Concluding Remarks

Individuals who abuse alcohol on a chronic basis are predisposed to the development of numerous diseases including cancer. GSH is a ubiquitous tripeptide that functions as a major cellular antioxidant and redox-buffering molecule. The transsulfuration

pathway metabolically connects GSH biosynthesis with cellular transmethylation. Chronic alcohol consumption results in depletion of the cellular GSH pool and inhibition of cellular transsulfuration/transmethylation, which are key pathogenic events involved in alcohol-associated tissue injury and carcinogenesis. Molecular details of these processes are yet to be defined. Therapeutic strategies targeted at improving these metabolic changes are inconclusive and warrant further studies.

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Chapter 4
Fatty Liver Disease and Hepatocellular Carcinoma: The Pathologist's View

Dina G. Tiniakos, João Maurício, and Helen L. Reeves

Abstract Chronic alcohol misuse and progressed nonalcoholic fatty liver disease (NAFLD) due to the metabolic syndrome and resulting to nonalcoholic steatohepatitis (NASH) are prime causes of hepatocellular carcinoma (HCC) in Western industrialized countries. The incidence of HCC in NASH-cirrhosis is lower than that of HCC occuring in HCV-related or alcoholic cirrhosis. Up to 20% of cases of alcohol-associated HCC may develop in pre-cirrhotic liver while HCC is also increasingly recognised in pre-cirrhotic NASH raising questions on appropriate surveillance measures for these patient populations. The recently described steatohepatitic subtype of HCC presents with higher frequency in NAFLD compared to alcoholic liver disease (ALD) patients. This review will mainly focus on histopathology and summarize current data on the epidemiology, pathogenesis, diagnosis and management of NAFLD- and ALD-related HCC.

Keywords Alcoholic · Nonalcoholic · Fatty Liver Disease · Steatohepatitis · Hepatocellular Carcinoma · Histopathology · Pathogenesis · Diagnosis

4.1 Introduction

Hepatocellular carcinoma (HCC) accounts for 70–85% of the total primary liver cancer burden and it usually arises in a background of chronic liver disease of hepatitis B virus (HBV), hepatitis C virus (HCV) or alcoholic aetiology [43, 46]. It is the fifth most common cancer in men and the ninth most common one in women. In 2012, 782,000 cases were estimated to have occurred globally, with 83% of these

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in less developed regions. HCC was the second most common cause of cancer-related death worldwide, with approximately 746, 000 deaths (9.1% of all cancer deaths in 2012). The prognosis for the majority of those affected with primary liver cancer is poor, with an overall mortality to incidence ratio of 0.95 [21, 46]. Most HCC cases (>75%) occur in Southeast Asia and sub-Saharan Africa, while Southern European countries have intermediate incidence rates and North/South America and Northern Europe present the lowest incidence rates (<5 per 100,000 individuals) [31]. HBV and HCV infections are the main aetiological factors of cirrhosis and HCC in Southeast Asia and sub-Saharan Africa. Chronic alcohol misuse and progressed nonalcoholic fatty liver disease (NAFLD) due to the metabolic syndrome and resulting to nonalcoholic steatohepatitis (NASH) are the prime causes of HCC in Western industrialized countries [69].

4.2 Epidemiology

4.2.1 ALD-related HCC

Hepatocellular carcinoma (HCC) reportedly develops in 5–15% of alcoholic patients with cirrhosis [35]. Alcoholic liver disease (ALD) is the commonest aetiology of HCC in industrialized countries, being responsible for 32–45% of cases [47]. A population-based USA study of nearly 7000 cases of HCC and > 250,000 controls found the risk of HCC (odds ratio – OR) to increase 4-fold in ALD patients and 2.5-fold in patients with NAFLD-associated diabetes and/or obesity [77].

Alcohol is also a recognized potentiating factor for HCC development in patients with chronic HCV-infection [25, 64, 76]. The 10-year cumulative occurrence rate of HCC in HCV-infected patients with alcoholic cirrhosis drinking >120 g alcohol per day is 80.7%, in contrast to 18.5% in alcoholic cirrhotics without evidence of HCV infection and 56.5% in non-drinkers with HCV-related cirrhosis [82]. Chronic alcohol consumption >80 g/day for >10 years increases the risk of HCC almost five-fold; chronic hepatitis C (CHC) patients who drink alcohol have double the risk of developing HCC compared to non-drinking HCV-infected patients [47]. A hospital-based, case-control study led by Hassan et al. [25] and involving 115 HCC patients and 230 non-liver cancer controls showed significant synergy between heavy alcohol consumption and CHC (OR 53.9) and diabetes mellitus (OR 9.9).

Data from human studies on the association between HBV infection and alcohol consumption are limited. In a French study evaluating the mortality related to HCV and HBV infections in 2001, 95% of HCV-infected patients who died had cirrhosis and 33% had HCC; 35% had reported excessive alcohol consumption. Similarly, in the HBV infection group, 93% and 35% of the individuals had cirrhosis had HCC, respectively and 5% had a history of excessive alcohol use. The mean age at death of both HCV- and HCV-infected patients who drunk excessively was significantly lower compared to non-drinkers or patients who drank modestly. Human immunodeficiency virus (HIV) infection was also a significant co-factor [42]. The risk of HCC development in ALD is increased if iron overload is also present [26].

4.2.2 NAFLD-related HCC

NASH-related cirrhosis may be complicated by HCC [6, 63, 68]. In NAFLD, older age, severity of insulin resistance and diabetes, and iron overload reportedly predispose to HCC [54]. Studies from various geographical areas have shown that the incidence of HCC in NASH-cirrhosis is lower than that of HCC developing in HCV-related or alcoholic cirrhosis [5, 32, 50]. Similarly, the risk of developing HCC is lower in NASH-related cirrhosis (1.3–2.4-fold) compared to that of HCV-cirrhosis (13–19-fold). However, in the last decades, the incidence of NASH-related HCC has been increasing worldwide, possibly as a consequence of the obesity and type 2 diabetes epidemic [57, 68]. In the United States, NAFLD is the fastest growing aetiology of HCC, especially among patients listed for liver transplantation [58, 79], while the number of NAFLD-associated HCC cases increases at a rate of approximately 9% per year [87]. In ALD patients, obesity and the metabolic syndrome appear to increase the incidence and mortality of HCC [14]. Pais et al. [52] have shown that the risk of HCC is higher in patients with alcohol-related cirrhosis in need for liver transplantation who also have NAFLD. In NAFLD patients, moderate alcohol use may potentiate the development of HCC [5].

A study by Marrero et al. [44] stated that NAFLD is the causal aspect of 13–38.2% of patients presenting with HCC not linked to viral infection or alcohol. In Northeast England, the overall incidence of HCC increased 1.8-fold from 2000 to 2010, with the astounding realisation that there was a > 10-fold increase in NAFLD-related HCC, accounting for 34.8% of all the cases in 2010. NAFLD thus became the most common background aetiology in this region [17]. Furthermore, irrespective of the underlying aetiology of HCC associated liver disease, over 50% of patients had type 2 diabetes and two thirds had either type 2 diabetes and/or obesity, defined as a body mass index >30 [17].

The prevalence of HCC is approximately 0.5% in nonalcoholic steatosis and 2.8% in NASH [68]. Studies in Japanese patients indicate that a greater length of follow-up may be necessary for determining the true prevalence of HCC [32]. According to a recent meta-analysis, the annual incidence of HCC in NAFLD patients is 0.44 per 1000 person-years, while in patients with NASH it rises to 5.29 per 1000 person-years [88]. In one of the first follow-up studies of NAFLD patients, HCC was reported in only one patient [55]. Shimada et al. [63] reported that 13 (7.3%) out of 82 cases of biopsy-proven NASH had cirrhosis and six of these (47% of cirrhotics) had HCC. In the same year, Bugianesi et al. [10] found that 44 out of 641 cirrhosis-associated HCC arose in cryptogenic cirrhosis, with cryptogenic cirrhotics having a higher prevalence of obesity, diabetes, markers of insulin resistance and increased triglycerides. This was the first study proposing the inclusion of cryptogenic cirrhosis and HCC in the natural history of NASH. In two subsequent studies, the incidence of HCC in patients with cryptogenic cirrhosis ranged from 18% [44] to 27% [56]. In both these studies, associated clinical features of metabolic syndrome implicated NAFLD as the underlying aetiology of cryptogenic cirrhosis.

4.3 Pathogenesis

4.3.1 ALD-related HCC

As mentioned above, HCC frequently arises in patients with a combination of alcoholic and viral liver disease related to HBV and/or HCV [33], but is also often described in patients without evidence of hepatotropic viral infection [81]. Alcohol is known to cause genetic alterations [34] but it can also act as a co-carcinogen by inducing the hepatic microsomal isoenzyme cytochrome P450 2E1 (CYP2E1), leading to activation of pro-carcinogens present in tobacco smoke, alcoholic beverages and food [22, 24, 69]. Alcohol, tobacco and obesity have been shown to be independent and synergistic risk factors for HCC development in patients with cirrhosis [45].

The mechanisms leading to HCC in either ALD or NAFLD background are notably similar. In ALD, there are several possible mechanisms by which alcohol can drive the development of HCC. These comprise, in addition to CYP2E1 induction that may activate pro-carcinogens as mentioned above, dietary or environmental carcinogens ingested alongside alcoholic drinks, toxicity of acetaldehyde, intensified lipid peroxidation due to reactive oxygen species (ROS), growth factor and cytokine milieu, deregulated immune responses, and DNA lesions caused by oxidative stress by-products [69].

Genetic factors can also play a role regarding predisposition for the development of HCC. A sequence variation within the gene coding for patatin-like phospholipase domain-containing protein 3 (PNPLA3, p.I148M) was shown to modify steatosis, necroinflammation and fibrosis in ALD [70]. Similar to PNPLA3, there may be a role for a transmembrane 6 superfamily member 2 (TM6SF2) gene variant across the ALD spectrum from steatosis, through cirrhosis to HCC [4]. Other polymorphisms implicated in the development of ALD-related HCC include genes involved in ROS formation (myeloperoxidase and superoxide dismutase 2) [49] and in inflammation (CCL5) [13].

Chronic alcohol use can also modulate microRNAs (miRNA/miR) expression influencing ALD progression [71]. For instance, miR-212 is involved in alcohol-induced gut permeability [72], miR-217 is implicated in steatosis via regulation of SIRT1 [86] and miR-199 is associated with an increase in endothelin-1 and hypoxia-inducible factor-1α, which play vital roles in inflammation and steatosis [85]. Several abnormally expressed miRNAs have been reported in HCC, including upregulation of miR-221, miR-21, miR-22 and miR-517a and downregulation of miR-29, miR-24a, miR-26a, miR15-a/b, miR-150, miR-195, miR-122, miR-20 family, miR-124 and let-7 family [71].

4.3.2 NAFLD-related HCC

Recent reviews have focused on mechanisms of hepatocarcinogenesis in NAFLD [43, 57, 89]. Fatty liver shows increased susceptibility to lipid peroxidation with subsequent production of free radicals that may cause DNA mutations. In obesity, fatty liver may be susceptible to carcinogens as a result of impaired ATP production, defective autophagy mechanisms, deregulation of energy and/or hormonal balance, hypoxia and systemic inflammation. In NAFLD, increased susceptibility of the steatotic liver to carcinogenic insults may be related to metabolic derangements related to the 'metabolic syndrome', hyperinsulinemia and the presence of insulin-like growth factor receptors in HCC, the systemic effects of deranged cytokines and adipokines, immune dysregulation and alteration in gut microbiota [43, 57, 89]. Genetic factors may also be responsible for increasing the risk of HCC in NAFLD patients [4]. Carriers of the PNPLA3 p.I148M variant are known to be at increased risk of progressive fibrosis and steatohepatitis of none alcoholic or alcoholic aetiology [59, 67]. Recently, a strong association has emerged between the common PNPLA3 p.I148M variant and the risk of developing HCC in NAFLD patients [38, 39, 65], reporting that the variant increased 3-fold the risk of progression to NASH and, most notably, 12-fold that of developing HCC [38, 39]. Another genetic variant, rs58542926 in *TM6SF2*, was found to be linked with NAFLD-related HCC in univariate analysis [38, 39].

In experimental models of hepatocarcinogenesis steatosis alone is not sufficient for HCC development [57]. Additional inflammation may be necessary as shown in mice under prolonged choline-deficient high fat diet that developed spontaneous HCC [78]. Alterations in the pro-inflammatory nuclear factor kappa B (NF-κB) signalling may play a significant role [57]. In a non-obese inbred mouse model with spontaneous fatty liver and steatohepatitis, hepatocellular adenomas and HCC emerge with time in up to 40% of male mice but <10% of female mice supporting gender predilection in HCC development [66].

4.4 Diagnosis

HCC is the only major cancer in which diagnosis and indication for treatment are not regularly established by histology. If a liver mass/nodule is detected on ultrasound then the most trustworthy imaging diagnostic tools for the detection of HCC are four-phase computed tomography (CT) and/or dynamic contrast enhanced magnetic resonance imaging (MRI). According to current guidelines [18], diagnosis of HCC in cirrhotic liver of any aetiology can be established if a mass > 2 cm shows specific imaging pattern (arterial hyper-enhancement followed by contrast washout in the venous/delayed phase) with one of the above mentioned techniques (positive predictive value and specificity >90–95%); in masses measuring 1–2 cm use of two imaging techniques (CT and MRI) is required and the accuracy of non-invasive

diagnosis is 73–88% [41]; a focal hepatic mass with atypical imaging characteristics, or a focal hepatic mass detected in a non-cirrhotic liver, should undergo biopsy [15, 20]. However, imaging diagnostic quality outside tertiary centres may not be as expertly assessed as the data which formed the basis of the guidelines, which was generated in carefully controlled and supervised multicentre trials. Although many cases of suspected HCC are referred to specialist centres for expert review, one recent study suggested that approximately 20% of presumed HCC nodules are incorrectly diagnosed by non-invasive techniques [51]. α-fetoprotein (AFP) serum levels >200 ng/mL have high specificity for HCC diagnosis in patients with cirrhosis and radiologic evidence of focal hepatic lesions, although AFP serum testing is not universally advocated owing to poor sensitivity – particularly in cases with early stage disease. Combination serum tests have been suggested, but have yet to impact clinical practice [7, 8, 29].

The specificity of liver biopsy for HCC diagnosis is 100%, with a superior overall sensitivity of 86–93%, although in nodules <1 cm the sensitivity falls to 83%. In approximately 2–11% of the cases a diagnosis cannot be made because of specimen inadequacy [48]. Immunohistochemical staining for glypican-3, heat-shock protein-70 and glutamine synthetase may aid diagnosis when conventional histology is not conclusive [15]. Liver biopsy in suspected HCC offers in addition to superior diagnostic sensitivity and specificity, precise histological typing/subtyping, prognostic and predictive information, and data for molecular classification. Molecular signatures from gene expression profiling based on HCC tissue material may be used in the future as biomarkers for prognosis and/or treatment stratification in HCC following careful validation [16, 40, 75].

The diagnosis of HCC frequently occurs at a late stage, a fact that may be explained by underutilisation of surveillance, delayed follow-up and suboptimal effectiveness of surveillance tests [31].

4.5 Histopathology

4.5.1 ALD-related HCC

HCC in ALD usually develops in a background of macronodular cirrhosis [35] (Fig. 4.1). However, up to 20% of cases of alcohol-associated HCC may also develop in precirrhotic liver disease [26]. Generally, HCC has acinar, pseudoglandular, trabecular and/or compact growth patterns, frequently with multiple histological patterns seen within a single tumour. Intracellular Mallory-Denk bodies (MDB) are frequent within ALD-related HCC and the incidence of HCC is significantly higher in ALD-cirrhosis with MDBs than without [69]. MDB contain p62 and other proteins, including keratins 8 and 18 and ubiquitin. p62 is an autophagy substrate and its overexpression is considered a marker of impaired autophagy implicated in the development of human HCC [2]. In the background surrounding liver, ALD

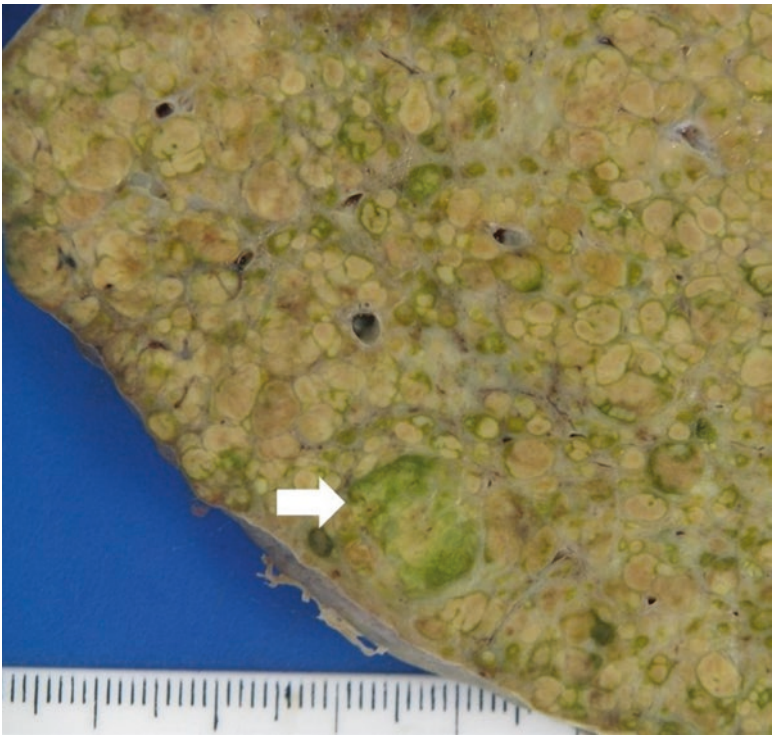

Fig. 4.1 Gross appearance of hepatocellular carcinoma (*arrow*) in segment VII of an explant liver with mixed micronodular and macronodular alcoholic cirrhosis

shares several histological features with NAFLD, such as steatosis and mixed parenchymal inflammation, and in the presence of steatohepatitis hepatocyte ballooning with or without Mallory-Denk bodies (MDB) and sinudoidal/pericellular fibrosis with a perivenular predominance [31, 74].

Dysplastic nodules in cirrhosis may be the precursor lesion for HCC in the multistage process of hepatocarcinogenesis [37, 73]. Dysplastic features, such as increased nuclear density ratio of >1.5, small-cell change and clear cell change have been associated with increased risk of progression of image-detected hepatic nodules to HCC in a prospective liver biopsy study [73].

The presence of large cell change, referring to groups of enlarged hepatocytes with mild nuclear pleomorphism, hyperchromasia and/or multinucleation, has been reported as an independent risk factor for HCC, with an estimated odds ratio of 3.3 [36]. However, in non-HBV related chronic liver disease, large cell change is not thought to be a dysplastic lesion and therefore a direct precursor of HCC; it is rather indicative of an increased risk for tumour development [30, 36, 37]. Histopathology reports of specimens from cirrhotic livers should always include a comment on the presence (or absence) of both small cell and large cell change [74].

Intrahepatic cholangiocarcinoma may occur in cirrhotic liver and its incidence is increasing [12]. Biliary intraepithelial neoplasia, a precursor of cholangiocarcinoma, has been described in the liver of patients with alcoholic cirrhosis, supporting its role in the development of biliary malignancy in this background [80].

4.5.2 NAFLD-related HCC

It is increasingly recognized that HCC may develop in non-cirrhotic liver in patients with NAFLD [11, 74] (Fig. 4.2). The first three cases were reported in 2008, in patients with features of the metabolic syndrome and steatosis without NASH or significant fibrosis [23]. In 2009, Paradis et al. reported 31 HCC arising in non-cirrhotic NAFLD patients with metabolic syndrome; of these, only one patient had NASH and 65% had mild fibrosis. Alexander et al. [3] have studied 157 patients with non-cirrhotic HCC and showed a strong association with the presence of NAFLD in the background liver. Compared to HCC in NAFLD-cirrhosis, tumours in non-cirrhotic NAFLD are usually larger and of lower histologic grade [53]. Male gender is a risk factor for HCC in non-cirrhotic NASH-related HCC in cohorts from France [53] and Japan [83, 84]. Other risk factors include type 2 diabetes and pre-existing hepatocellular adenoma [53]. In Japan, 21–28% of NAFLD-related HCC developed in patients without advanced fibrosis. Currently, screening and surveillance of patients with advanced NAFLD are reserved only for those with cirrhosis,

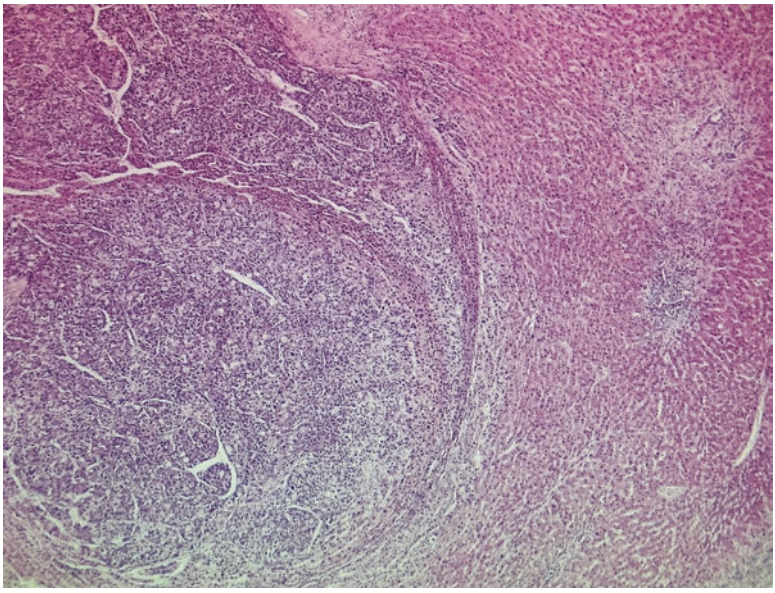

Fig. 4.2 Hepatocellular carcinoma (*left*) in non-cirrhotic liver (H-E, x100)

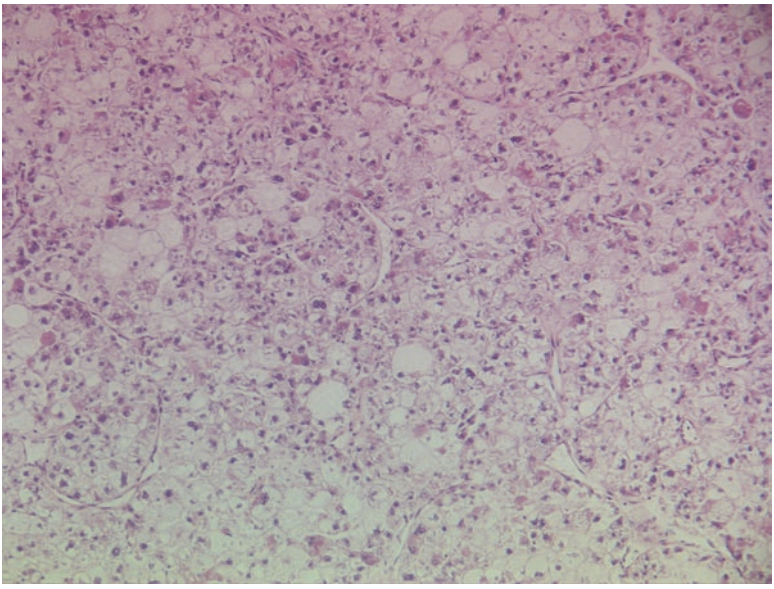

Fig. 4.3 Steatohepatitic hepatocellular carcinoma: many tumour cells are steatotic and some are ballooned and contain Mallory-Denk bodies (H-E, x200)

raising concern over the management of non-cirrhotic patients [6, 17, 27]. The insufficiency of early detection screening methods in non-cirrhotic NAFLD may explain the fact that most NAFLD-related HCC present at an advanced stage [79].

A special HCC subgroup with steatohepatitic morphology (SH-HCC) has recently been described (Fig. 4.3) [60, 61], presenting a higher frequency in NAFLD compared to ALD [28, 62]. In SH-HCC, >5% of tumour cells contain fat, there is widespread ballooning with or without MDB, interstitial fibrosis and foci of mixed inflammation, including neutrophils. Steatohepatitic features have been reported in 13.5–36% of HCC and are more commonly seen in tumours from patients with metabolic risk factors and steatosis or steatohepatitis in the surrounding non-neoplastic liver [3, 28, 60–62]. The presence of steatohepatitic morphology does not affect HCC prognosis [28, 62].

4.6 Management

Management decisions for HCC, independent of aetiology, are widely based on the Barcelona Clinic for Liver Cancer (BCLC) staging system - which incorporates an assessment of patient liver function and performance status in addition to tumour burden, linked to a treatment algorithm [18]. BCLC staging enables stratification of patients to those fit for potentially curative treatments (resection, transplantation and ablation) and those better served by transarterial chemoembolization or systemic

therapies such as [31]. Nowadays, curative therapies can improve survival in patients diagnosed at an early stage and provide a possible long-term cure. Surgical resection is the first-line option for patients with solitary HCC without clinically relevant portal hypertension; patients with portal hypertension and early stage HCC defined as within Milan criteria (1 lesion <5 cm or 3 lesions <3 cm) are considered for transplantation. Patients with intermediate stage HCC may benefit from hepatic arterial therapies delivering treatment preferentially to the tumour rather than non-tumour tissues - typically an embolising agent in combination with doxorubicin or cisplatin. Longer term follow up of these patients has yet to be reported and presently, the lack of tissue assessment pre-transplant has hampered the identification and validation of predictive biomarkers with the potential to identify patients with downstaged disease who are more likely to benefit from transplantation. Patients diagnosed at advanced stages may benefit from sorafenib, a multikinase inhibitor with antiangiogenic and anti-proliferative effects [15]. Sorafenib was the first and still is the only approved systemic therapy targeting pathways involved in hepatocarcinogenesis as the majority of subsequent phase III randomised trials have with molecular inhibitors have failed [16]. The tide may have turned recently, as a sorafenib-like multikinase inhibitor has been shown to have benefit 2nd in patients whose tumours progress on sorafenib [9]. Enrichment trials stratifying patients expressing c-MET on liver biopsy to treatment or not with a MET inhibitor are awaited (www.clinicaltrials.gov). Furthermore, early studies with immune checkpoint blockade, targeting the tumour inhibition of cytotoxic T cell responses, have shown promise in some patients with HCC [1, 19]. The need to identify biomarkers that will aid patient stratification to one therapy over another may yet drive the need for tumour biopsy in the not too distant future.

4.7 Epilogue

HCC in the setting of NAFLD is increasing and associations with the metabolic syndrome, diabetes and obesity are well established. The recently described steatohepatitic subtype of HCC presents with higher frequency in NAFLD compared to ALD patients. In contrast to ALD, HCC in NAFLD frequently develops in non-cirrhotic liver raising questions on appropriate surveillance measures for this population. On the other hand, surveillance for HCC in alcoholics with cirrhosis is less effective because of socioeconomic reasons. Patient and tumor characteristics in NAFLD-associated HCC are different compared to HCC of other aetiology, with older age and cardiovascular disease posing problems in therapeutic decisions and limiting available treatment choice. However, early stage HCC in NAFLD-patients has an excellent outcome and curative therapy should be applied in suitable patients. Prevention of obesity and underlying metabolic conditions, early HCC diagnosis through targeted surveillance programs and more effective treatment modalities are clearly needed for reducing the burden and improving the outcome of NAFLD-related HCC. The burden of ALD-related HCC is entirely preventable by reducing the prevalence of harmful and/or hazardous alcohol use.

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Chapter 5
Alcoholic Liver Disease Accelerates Early Hepatocellular Cancer in a Mouse Model

Gyongyi Szabo

Abstract HCC is a rapidly increasing cancer worldwide. Most HCC rises in the setting of chronic and advanced liver disease caused by viral hepatitis, alcohol use, non-alcoholic liver disease or their combination. We found that in the mouse model, alcohol alone does not induce HCC, however, it can promote HCC development after a carcinogen exposure. Multiple mechanisms are involved in carcinogenesis and alcohol affects many of those including epithelial-mesenchymal transition, cancer stem marker expression and inflammation as evidenced in our HCC model.

Keywords Inflammation · miR-122 · DEN · Macrophage polarization · Neutrophil leukocyte · Biliary cyst · Alpha fetoprotein · Stemness · Hypoxia-inducible factor-1alpha

5.1 Introduction

Hepatocellular cancer (HCC) is the fastest growing cancer in the US and worldwide [1, 9, 25]. However, therapeutic options and effectiveness of therapy are limited for HCC. Epidemiology and natural history studies have found that most HCC develops in patients with cirrhosis and end-stage liver disease and thus, all factors that lead to liver cirrhosis are correlated with development of HCC. The most frequent etiology of liver disease leading to HCC include chronic viral hepatitis with hepatitis C or hepatitis B infection, alcoholic liver disease and non-alcoholic steatohepatitis (NASH) [1, 9, 19, 25]. Importantly, recent studies suggest HCC development in NASH without the presence of cirrhosis [13]. In cases of chronic HBV infection, HCC development can also be seen in the absence of cirrhosis and this is largely attributed to the fact that HCV as a DNA virus, can integrate into the host genome thereby causing direct carcinogenic effects [18].

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Chronic alcohol use alone increases the risk of HCC particularly in men and alcohol significantly accelerates HCC in patients with chronic HCV infection suggesting that alcohol can act as a co-factor in the presence of other tumorigenic factors [19, 24].

5.2 New Model of HCC in Mice with Alcoholic Liver Disease

Animal models of HCC have been developed with the aims to provide a model of human HCC, accelerate mechanistic understanding of HCC, and provide a tool for preclinical testing of new potential therapeutics for HCC [5, 14]. The classical tumor inducer, DEN, was shown to lead to HCC when injected into neonatal mice. In addition to HCC, however, these mice develop other types of cancers as well indicating that this model is not a liver-specific carcinogen. Using the DEN model, McKillop's group reported increased tumor development when mice received chronic low doses of alcohol in the drinking water [6]. In this model, mice that received alcohol and no DEN, showed no liver damage that is very different from the human condition where alcohol-related HCC develops in the liver with alcoholic hepatitis and/or alcoholic cirrhosis [24].

To mimic the underlying element of human HCC, alcoholic liver disease, we have developed a new model where DEN was combined with chronic alcohol administration that results in features of human alcoholic liver disease [2]. In this new alcohol-HCC model, 4-week old male C57bl6 mice received weekly doses of DEN (75 mg) for three doses followed by another 3 weekly doses of DEN (100 mg) as described [3, 6]. Alcohol feeding was started on week 6 of DEN administration and the Lieber-DeCarli alcohol (4% ethanol) or pair-fed diet was administered for 7 weeks [2, 3].

5.3 Liver Damage, Histology and Tumor Characteristics

Even the first DEN administration induced liver damage as indicated by increased serum ALT levels and the ALT increase was sustained throughout the DEN administration indicating the presence of alcoholic liver disease [2]. Chronic alcohol feeding with the Lieber-DeCarli diet resulted in significant increase in serum ALT and bilirubin levels compared to pair-fed diet. The combination of alcohol and DEN

Table 5.1 Liver fibrosis markers

	Pair-fed		Alcohol-fed	
	No DEN	DEN	No DEN	DEN
ALT	–	↑	↑	↑↑
Sirus red staining	–	↑	↑	↑↑↑
α SMA	–	↑	–	↑↑
TGFβ	–	↑	–	↑↑
Pro-/collagen-1	–	↑	↑	↑↑↑

induced the highest levels of ALT that was significantly greater than ALT increased by DEN or alcohol alone suggesting an additive effect (Table 5.1). CyP2E1, that is involved in the metabolism of alcohol was increased by alcohol and DEN, respectively; however, alcohol plus DEN administration showed no additive effect at the end of the 7 weeks of alcohol feeding [2].

Liver triglyceride measurements, hematoxylin eosin and oil-red-O staining of livers revealed fatty liver disease after alcohol alone administration compared to pair-fed controls. DEN alone resulted in no significant steatosis and, interestingly, livers from DEN plus alcohol-fed mice had less steatosis compared to alcohol alone treated mice [2, 3].

Consistent with the greater extent of liver damage in DEN plus alcohol fed mice, we found the most extent of liver fibrosis on Sirius red staining in this group compared to others (Table 5.1). Markers of liver fibrosis including alpha-smooth muscle actin, TGBß and pro-collagen1 expression levels were increased by alcohol or DEN alone and showed significantly higher levels in DEN plus alcohol treated animals compared to any other groups (Table 5.1) [2, 3]. It is notable that in human disease progression of alcoholic liver disease to cirrhosis, the extent of steatosis also shows a decreasing trend compared to early alcoholic steatohepatitis.

Hepatic tumor development was found in mice with DEN plus alcohol treatment. DEN alone resulted in no hepatic dysplastic nodules indicating early HCC but alcohol pus DEN treated mice had significantly higher numbers of hepatic nodules [2, 3]. We also observed biliary cysts in DEN treated mice (average of cysts/ livers) that was significantly higher in alcohol plus DEN treated mice [2, 3]. Hepatic tumor development was also confirmed by liver MRI analysis [2, 3]. Serum alpha fetoprotein, a marker of hepatocyte proliferation and HCC, was significantly increased by alcohol and DEN alone, respectively, with a synergistic increase after DEN plus alcohol combination (Table 5.2).

Table 5.2 Markers of stemness and carcinogenesis

	Pair-fed	Pair-fed	Alcohol-fed	Alcohol-fed
	No DEN	DEN	No DEN	DEN
AFP	—	↑	↑	↑
CYCLIN D	—	—	—	↑
P53	—	↑	↑	↑↑
Vimentin	—	↑	↑	↑↑
N-cathedrin	—	↑	—	↑↑↑
E-cathedrin	—	—	↓	↓↓
Gli1	—	↑	—	↑↑↑
Ccnd2	—	↑	—	↑↑
Osteopontin	—	↑	—	↑↑↑
CD44	—	↑	—	↑↑
Shh	—	—	—	↑
CD133	—	↑	↑	↑↑↑
Nanog	—	↑	—	↑↑

5.4 Role of Inflammation in HCC

A critical feature of alcoholic liver disease and alcoholic hepatitis is recruitment and activation of inflammatory cells in the liver [11, 22]. Furthermore, chronic inflammation has been identified as a significant driving factor in development of hepatocellular cancer [12]. Both DEN and chronic alcohol feeding increased the expression of pro-inflammatory cytokines, TNFα, IL-6, MCP-1, IL-17a at the mRNA and protein levels in the liver [2]. The combination of DEN plus alcohol was additive in MCP-1 and IL-17 protein induction (Table 5.3). This correlates with findings in human HCC where increased serum levels of IL-17 and MCP-1 were associated with HCC [15, 17].

Alcohol alone and, to a significantly greater extent in combination with DEN, resulted in increased neutrophil leukocyte recruitment in the liver indicated by increased mRNA levels of Ly6G, E-selectin and myeloperoxidase; the latte was confirmed by immunohistochemistry staining [2] (Table 5.3). Macrophages were also activated in the liver after chronic alcohol feeding indicate by increased F4/80 and CD68 mRNA expression (Table 5.3). The significantly higher CD68 expression after the combination of alcohol plus DEN indicated significantly increased recruitment of macrophages to the liver. Macrophages can express different phenotypes in response to various stimuli from their tissue environment [17]. The spectrum of MØ phenotypes includes pro-inflammatory macrophages that produces mediators, cytokines in tissue inflammation while the opposite spectrum is "repair" macrophages that participate in tissue remodeling and resolution of inflammation [15, 17]. We found evidence for the presence of both of these phenotypes after alcohol, DEN or their combination (Table 5.3). We noted that the combination of alcohol plus DEN preferentially increased the expression of IL-10 and CD206 that are markers of

Table 5.3 Markers of inflammation in early HCC accelerated by ALD

	Pair-fed No DEN	Pair-fed DEN	Alcohol-fed No DEN	Alcohol-fed DEN
TNFα	—	↑	↑	↑↑
IL-6		↑	↑	↑↑
MCP-1	—	↑	↑	↑↑
IL-17A	—	↑	↑	↑↑
iNOS	—	↑	↑	↑↑
ARG1	—	↑	—	↑
IL-10	—	↑	—	↑
F4/80	—	↑	—	↑
CD68	—	↑	—	↑↑
CD163	—	↑	—	↑
CD206	—	↑	—	↑↑
Ly6G	—	↑	—	↑↑
MPO	—	↑	—	↑↑
E-selectin	—	↑	—	↑↑

tumor-associated macrophages (TAMs) (Table 5.3). These results suggested that in alcoholic liver disease and in alcohol-induced HCC, both M1 and M2-type macrophages are induced and further understanding of the specific role of these MØ phenotypes in HCC might be important in future therapeutic interventions.

5.5 Markers of Stemness and Carcinogenesis

An increased frequency of liver lesions indicating early HCC was detected by MRI, and AFP in DEN treated plus alcohol-fed mice compared to DEN alone, alcohol alone or pair-fed controls [2, 3]. AFP increase was present in the serum as well as in the liver as determined by immunohistochemistry staining (Table 5.2). AFP is expressed at increased levels in proliferating and de-differentiated hepatocytes and this notion was supported by increased expression of PCNA on liver histology [2]. Recent reports suggest that HCC development occurs from the growth of a small population of cancer stem cells or tumor initiating cells (TICs). These are characterized by expression of the molecular stemness markers, CD133 and nanog [16, 23]. While both CD133 and nanog mRNA levels were increased by alcohol or DEN alone, there was a significant increase in the livers with DEN induction followed by alcohol treatment compared to any other experimental groups (Table 5.2). This correlated with increased liver expression of cytokeratin 7 and cytokeratin 9 [3]. The tumor markers, cyclin D1, p53 as well as markers of epithelial mesenchymal transition (EMT), vimentin and n-cathedrin were significantly increased in livers with the highest tumor numbers after DEN plus alcohol treatment (Table 5.2).

The intracellular signaling pathways associated with HCC were also increased. We found significant increase in key elements of hedgehog signaling in DEN plus alcohol treated mice. The mRNA expression of Gli1, Ccnd2 and osteopontin were all significantly increased in livers after DEN plus chronic alcohol feeding compared to DEN or alcohol alone (Table 5.2) [3].

5.6 MicroRNA-122

MicroRNAs are 22 nucleotide small RNAs that regulate post-transcriptional gene expression via RNA silencing [4, 20]. Different microRNAs have been found to be up or downregulated in HCC [7, 10, 27]. In normal hepatocytes, miR-122 is expressed at high levels and it represents about 70% of the total miRNA pool in hepatocytes [4, 7, 10, 20, 27]. We found a significant reduction in liver miR-122 levels after DEN plus alcohol feeding, however, serum levels of miR-122 were significantly increased (Table 5.4) [3]. This suggested that in early HCC hepatocytes most likely release miR-122 into the circulation. While the biological significance of increased circulating miR-122 is yet to be defined, increase in circulating miRNA-122 deserves consideration as an early biomarker of liver injury and HCC [21]. Our

Table 5.4 MicroRNA-122 and targets

	Pair-fed No DEN	Pair-fed DEN	Alcohol-fed No DEN	Alcohol-fed DEN
Liver miR-122	—	↓	↓	↓↓
Serum miR-122	—	↑	—	↑↑
Ccng I	—	↑	↑	↑↑
Bclw	—	↑	—	↑↑
HIF1α	—	↑	—	↑↑
VEGF	—	—	—	↑

Table 5.5 Serum concentration

	ALT	AFP	CD133
miR-122	r = 0.7413	r = 0.6742	r = 0.5148
	P < 0.001	P < 0.0001	P < 0.01

previous study indicates that increase in serum miR-122 levels show a significant correlation with liver damage indicated by increased ALT, the tumor marker, alpha fetoprotein (AFP), and the EMT marker, CD133 (Table 5.5). Circulating miRNAs are being exploited as biomarkers and may serve as a "liquid biopsy" for HCC and liver disease [26].

The functional effect of decreased miR-122 in livers with early HCC was further evaluated in our studies and we found that the miR-122 target, hypoxia-inducible factor 1α was affected. HIF-1α was recently identified as a miR-122 target [8] and HIF-1α upregulation has received interest as a therapeutic target in HCC [27]. There was a significant increase in HIF-1α levels and DNA binding in livers with early HCC after DEN plus chronic alcohol feeding (Table 5.4) [3]. The increase in HIF-1α is important considering that the tumor microenvironment is often hypoxic providing another likely mechanism for upregulation of HIF-1α.

5.7 Summary and Relevance to Human Disease

HCC is a rapidly increasing cancer worldwide. Most HCC rises in the setting of chronic and advanced liver disease caused by viral hepatitis, alcohol use, non-alcoholic liver disease or their combination. We found that in the mouse model, alcohol alone does not induce HCC, however, it can promote HCC development after a carcinogen exposure. Multiple mechanisms are involved in carcinogenesis and alcohol affects many of those including epithelial-mesenchymal transition, cancer stem marker expression and inflammation as evidenced in our HCC model. It is important to note, that we found acceleration of HCC development in livers with alcoholic liver disease and not in mice that received alcohol without a carcinogen (DEN). Our data and that of others suggest that alcohol acts at multiple levels in acceleration of HCC including increasing inflammation in the liver, interfering with

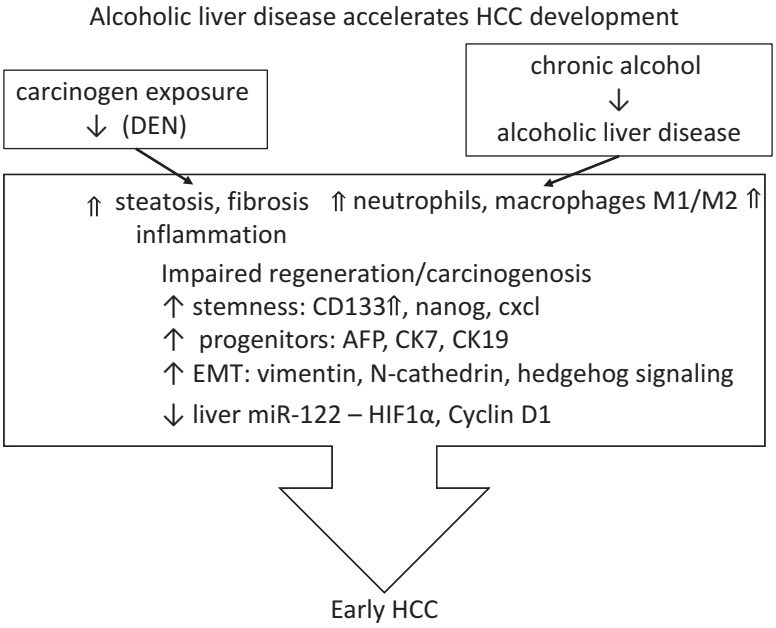

Fig. 5.1 Alcoholic liver disease accelerated HCC development in mice. Factors contributing include alcohol-induced steatosis, inflammation, macrophage and neutrophil activation in addition to DEN-induced carcinogenesis and impaired liver regeneration

liver regeneration, increasing stemness and EMT and decreasing liver mRNA levels (Summarized in Fig. 5.1). This is important when extrapolating to human disease because in humans only excessive chronic alcohol use is associated with HCC that results in liver cirrhosis and end-stage liver disease. Moderate alcohol use is not associated with HCC in humans unless some other chronic liver diseases is also present. Thus, alcohol appears to be a co-factor and not a primary initiator of HCC.

Similar to many other HCC models, there are some limitations of this new model as well. While human HCC develops in livers with alcoholic cirrhosis and typically not without significant fibrosis, alcoholic liver disease in mice does not progress to the same extent of fibrosis that is seen in human disease. However, we found that administration of DEN together with the prolonged alcohol feeding resulted in the greatest extent of liver fibrosis compared to alcohol or DEN alone. It remains to be determined whether human HCC is associated with the extent of the fibrosis/cirrhosis in ALD or with the time factor of alcohol-induced damage to hepatocytes and stem cells that also contribute to the development of cirrhosis. Studies with tumor stem cells indicate that alcohol-induced damage and modification of stem cells is a key factor in HCC development. Indeed, we found upregulation of cyclin D1 and p53 in the liver with DEN plus chronic alcohol treatment.

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Chapter 6
Chronic Ethanol Consumption and Generation of Etheno-DNA Adducts in Cancer-Prone Tissues

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Abstract Chronic ethanol consumption is a risk factor for several human cancers. A variety of mechanisms may contribute to this carcinogenic effect of alcohol including oxidative stress with the generation of reactive oxygen species (ROS), formed via inflammatory pathways or as byproducts of ethanol oxidation through cytochrome P4502E1 (CYP2E1). ROS may lead to lipidperoxidation (LPO) resulting in LPO-products such as 4-hydoxynonenal (4-HNE) or malondialdehyde. These compounds can react with DNA bases forming mutagenic and carcinogenic etheno-DNA adducts. Etheno-DNA adducts are generated in the liver (HepG2) cells over-expressing CYP2E1 when incubated with ethanol;and are inhibited by chlormethiazole. In liver biopsies etheno-DNA adducts correlated significantly with CYP2E1. Such a correlation was also found in the esophageal- and colorectal mucosa of alcoholics. Etheno-DNA adducts also increased in liver biopsies from patients with non alcoholic

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steatohepatitis (NASH). In various animal models with fatty liver either induced by high fat diets or genetically modified such as in the obese Zucker rat, CYP2E1 is induced and paralleled by high levels of etheno DNA-adducts which may be modified by additional alcohol administration. As elevation of adduct levels in NASH children were already detected at a young age, these lesions may contribute to hepatocellular cancer development later in life. Together these data strongly implicate CYP2E1 as an important mediator for etheno-DNA adduct formation, and this detrimental DNA damage may act as a driving force for malignant disease progression.

Keywords Cytochrome P4502E1 · Etheno-DNA adducts · Reactive oxygen species · NASH · ALD · Esophageal Cancer · Colorectal Cancer

6.1 Introduction

There is strong evidence that oxidative stress related DNA damage is induced by known inherited and acquired cancer risk factors including inflammation [1–3]. Pro-mutagenic Lipidperoxidation (LPO)-derived DNA adducts are increased significantly in chronic pancreatitis [2], ulcerative colitis, Crohn's disease [2] as well as in viral-Hepatitis [2] and other types of chronic liver disease [4]. Two major etheno-DNA adducts 1,N6-etheno-2′-deoxyadenosine (εdA) and 3,N4-etheno-2′-deoxycytidine (εdC) were quantified as marker lesions and found to accumulate in target organs over time, paralleling progression to tumor development [1–3, 5, 6]. DNA repair and cellular apoptotic processes contribute to urinary excretion of etheno-desoxyribonucleosides, which offer a non-invasive approach to monitor LPO-related pathogenic processes in vivo [7].

In this article we review formation and significance of exocyclic etheno DNA adducts and their possible role in human and experimental carcinogenesis. Major emphasis, however, will be led on the effect of chronic ethanol consumption and the generation of these adducts in the liver and other extrahepatic tissues. Finally, the relevance of etheno DNA adducts in non-alcoholic fatty liver disease (NAFLD) is discussed.

6.2 Etheno-DNA Adducts: Formation and Significance

Upregulation and overexpression of stress response enzymes such as inducible nitric oxide synthase (iNOS), lipoxygenase (LOX) and possibly cyclooxygenase (COX)-2 in inflamed tissues proceeds malignant growth. Hereby a self-perpetuating stimulation of LPO, over-production of DNA-damaging ROS and reactive nitrogen species (RNS), as well as LPO-derived exocyclic-DNA adducts takes place, acting as a driving force to malignancy [2] (Abb.1).

This cascade of events was supported by rodent models and adduct-analysis of organ tissue/ biopsy samples from cancer-prone patients. In Swiss Jim-Lambert-mice

inflammatory-related NO overproduction was found to be associated with significant increased etheno-DNA formation; both could be inhibited by the administration of an iNOS inhibitor [8]. In a multistage mouse skin carcinogenesis model etheno-DNA adducts correlated with LOX-catalyzed tumor associated arachidonic acid metabolites [9]. Similarly, increased adduct levels were found in target tissues of Apc min (multiple intestinal neoplasia) mice [10, 11] as well as in cancer-prone patients with familial adenomatous polyposis (FAP) [12].

Induction of cytochrome P-450 2E1 as in alcoholic liver disease (ALD) and NAFLD may also result in ROS and etheno-DNA adduct formation [13, 14]. Although ethanol is primarily oxidized via alcohol dehydrogenase, a small percentage is metabolized via the microsomal ethanol oxidizing system (MEOS) which is CYP2E1 dependent. This pathway increases when ethanol is consumed chronically. Besides acetaldehyde, the first metabolite of ethanol oxidation, ROS are generated which trigger lipid peroxidation (LPO), leading to DNA adduction that likely participates in tumourigenesis (Fig. 6.1).

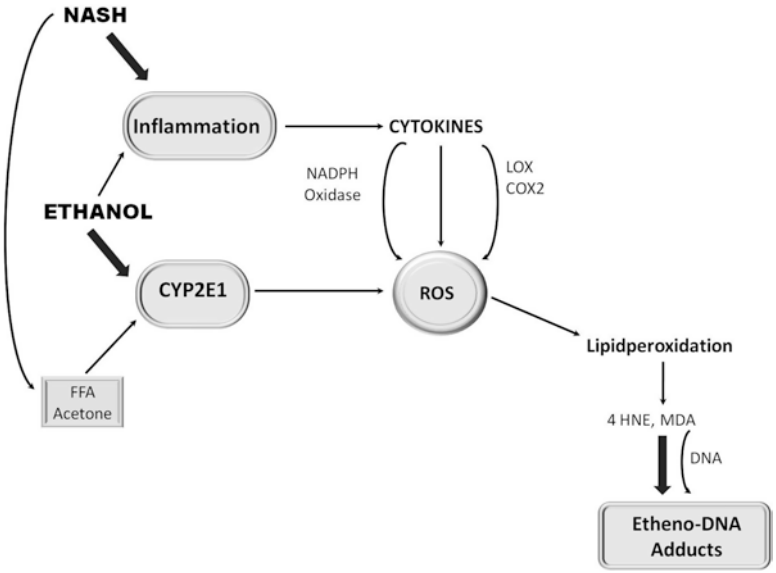

Fig. 6.1 Simplified pathophysiology of reactive oxygen species (ROS) and etheno DNA adduct formation. Inflammation driven cytokine secretion results among others in an activation of NADPH oxidase and via NFκB in an activation of lipoxygenase (LOX) and cyclooxygenase 2 (COX-2). As a result ROS are generated, which lead to lipidperoxidation with the occurrence of lipidperoxidation products such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). These adducts react with DNA bases to form exocyclic etheno-DNA adducts. Chronic alcohol consumption results in the induction of cytochrome P4502E1 (CYP2E1), which is involved in ethanol oxidation via the microsomal ethanol oxidizing pathway. During this reaction ROS is generated without inflammation. To a minor degree ethanol may result in ROS formation through inflammation (alcoholic hepatitis). On the other hand, in NASH ROS is primarily formed through inflammation and to a minor degree through C YP2E1 induction via acetone (diabetes mellitus) and/or free fatty acids (FFA)

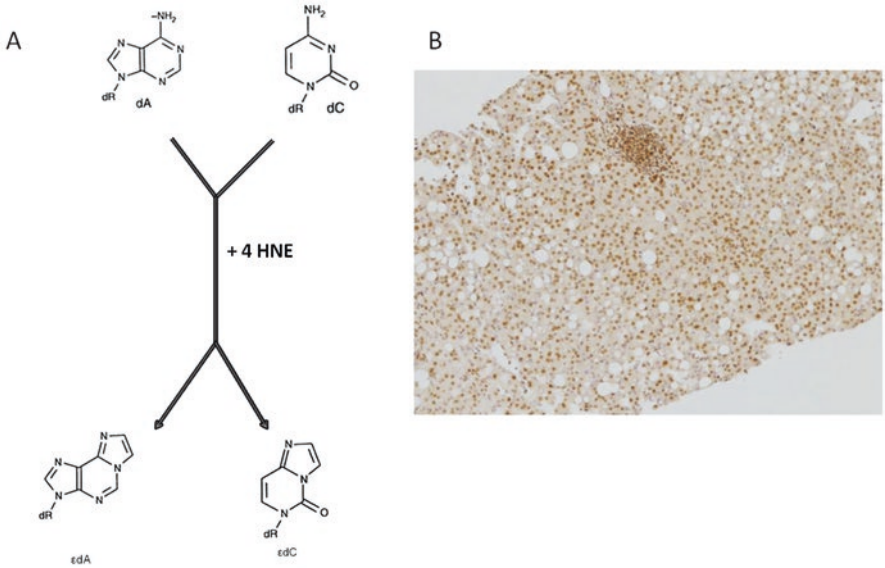

Fig. 6.2 1,N6-etheno-2′-deoxyadenosine(εdA), and 3, N4-etheno-2′-deoxycytidine (εdC), two important etheno-DNA adducts (**a**). Immunohistochemistry of εdA in the nuclei of hepatocytes in a patient with ALD (**b**)

Thus, current evidence supports the paradigm that cancer predisposing conditions (see above) lead to the ROS/RNS generation with subsequent LPO and production of by-products such as malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), 4-hydoxyhydroperoxy-2-nonenal (HPNE) (Fig. 6.2). These lipidperoxidation products react with DNA either directly or through bifunctional intermediates to form various promutagenic exocyclic etheno-DNA adducts [13]. LPO-products derived mainly from gamma-linoleic acid, include 4-HNE, a major LPO product and its electrophilic epoxy-, hydroperoxy-, and oxo-enal intermediates can react with the DNA bases A, C, and G. This yields the unsubstituted etheno-DNA adducts, 1,N6-etheno-2′-deoxyadenosine (εdA), 3,N4-etheno-2′-deoxycytidine (εdC), 1,N2-etheno-2′-deoxyguanosine (1,N2εdG), and N2,3-etheno-2′-deoxyguanosine (N2,3εdG). In addition, substituted base adducts are formed such as HNE-dG carrying a fatty acid chain residue. 2,N4-etheno-5-methyl-2′-deoxycytidine (ε5mdC), an endogenous LPO-derived adduct was recently identified in human tissue DNA, possibly playing a role in epigenetic mechanisms of carcinogenesis [1, 15–22]. DNA is also modified directly by ROS and RNS to yield 8-nitro-dG and 8-Oxo-dG [16]. All of these products in DNA changes have been detected in human specimens [4, 7, 23–26]. Exocyclic etheno-DNA adducts exhibit strong mutagenic properties in most organisms tested so far, producing various types of base pair substitution mutations and other types of genetic damage [27–32].

εdA can lead to AT → GC transition and AT → TA and AT → CG transversions [29, 30]. εdC cause CG → AT transversions and CG → TA transition [31, 32]. N2,3 εdA leads to GC → AT transition [32]. Incorporation of a single εdA in either DNA strand of HeLa cells showed a similar miscoding frequency and was more mutagenic than 8-oxo-dG [33].

Etheno adducts are poorly repaired in some tissues stressing their biological relevance [34]. Strong support that etheno-DNA adducts play a causal role in the initiation and progression of liver carcinogenesis comes from the formation of εdA and εdC in vivo by the human liver carcinogen vinyl chloride [35] and by the potent multiorgan, multispecies carcinogen urethane; hereby reaction with DNA occurs via their metabolic epoxy-intermediates [36]. The biological importance of etheno-DNA adducts is further stressed as they are preferentially formed in codon 249 of TP53 (which encodes p53), leading to a mutation that renders cells more resistant to apoptosis and provides them some growth advantage [37].

LPO-derived reactive products and their macromolecular interactions have been so far characterized primarily by in vitro studies, making it difficult, to pinpoint the main precursors and pathways involved in the generation of cancer-relevant DNA damage in human in vivo. For this reason, earlier studies analyzed εdA and εdC in human specimens to serve as a lead marker for other exocyclic adducts formed in vivo, but for which sensitive detection methods were not yet available at that time. Using ultrasensitive and specific detection methods [24], two miscoding etheno-DNA adducts εdA and εdC were first unequivocally identified in human DNA (Fig. 6.3) Subsequently, surgical tissue samples collected from “at risk” patients, i.e. affected by chronic inflammatory processes, persistent viral infections, iron storage- and alcohol-related diseases or exposed to inherited/acquired cancer risk factors were analyzed. Adduct levels increased 10–100-fold progressively in human cancer-prone organs including liver, bile duct, esophagus, colon and pancreas. Consistent results were also observed in rodent tumor models, that mimic human disease [3]. Taken together these data incriminate LPO-derived DNA adducts formed endogenously as strongly mutagenic and potentially cancer-causing lesions. The chemical structure of εdA and εdC as well as the immunohistochemical appearance of εdA in the liver of a patient with ALD is shown in Fig. 6.3.

6.3 Etheno-DNA Adducts in ALD and NAFLD: The Role of CYP2E1 Induction and Inflammation

Oxidative stress is a major pathogenetic factor in ALD and in NAFLD. In both diseases inflammatory driven oxidative stress occurs, which is predominant in non-alcoholic steatohepatitis (NASH) [13] as well as in alcoholic hepatitis (ASH), a clinical syndrome with high mortality [38]. In addition, CYP2E1 is found to be induced by chronic ethanol ingestion [39] as well as in NASH [40]. The intensity of ethanol mediated CYP2E1 induction differs interindividually [41]. In NASH, an

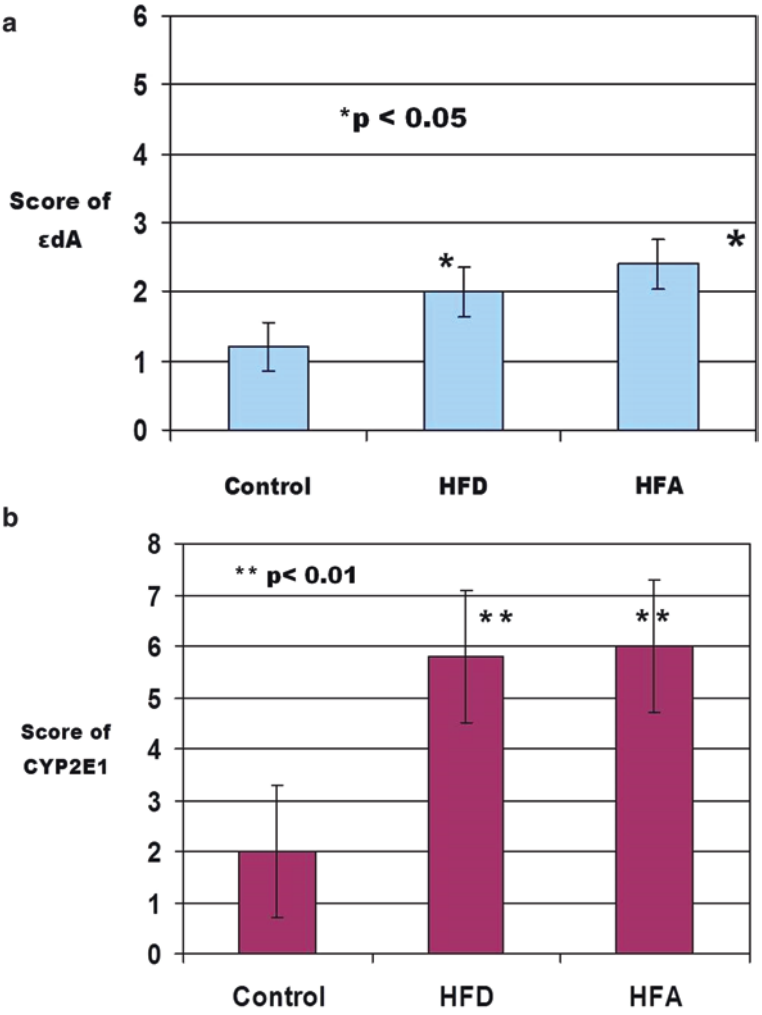

Fig. 6.3 Effect of a high fat diet with and without ethanol (16% of total calories on the level of εdA (**a**) and CYP2E1 (**b**). The high fat diet alone increased both εdA and CYP2E1 significantly, while the addition of ethanol did not further increase the two parameters. HFD = high fat diet, HFS = high fat diet plus alcohol

inflammatory liver disease associated primarily with the metabolic syndrome (overweight, diabetes mellitus, hypertension, and hypercholesterolemia) hepatic acetone (observed in diabetes mellitus) and free fatty acids (present in fatty liver) also induce CYP2E1, since there metabolism is catalyzed by CYP2E1 [42].

Ethanol metabolism through CYP2E1 generates not only acetaldehyde, but also ROS which can react with proteins and DNA affecting their structure and function. ROS can also initiate LPO which leads to formation of several byproducts such as

MDA and 4-HNE. After reaction with DNA bases exocyclic etheno DNA adducts are generated (Figs. 6.1 and 6.3). CYP2E1 induction in NASJH as compared to ALD was found to be less pronounced, whereas inflammation was predominant [13] This led to the assumption that DNA adduct formation in NASH is primarily driven by inflammatory processes, whilst in ALD, CYP2E1 induction is much stronger and inflammation generally milder. AH seems to be an exception, whereby in ALD etheno adducts are primarily formed via CYP2E1-mediated ROS formation.

6.4 Etheno DNA Adducts (εdA) in Alcohol Consuming Rodent Models and ALD Patients

Various animal experiments have underlined that in ALD CYP2E1 is responsible for the generation of ROS and DNA damage in disease causation: a) CYP2E1 knock-out mice do not develop ALD with the same severity as wild-type mice when they ingested alcohol for more than 4 weeks [43, 44]; b) inhibition of CYP2E1 by chlormethiazole (CMZ), a selective CYP2E1 inhibitor decreases ROS/RNS significantly, resulting in an inhibition of ALD [44, 45]; c) CYP2E1 knock-out mice also developed less oxidized DNA products as compared to wild type mice when both received ethanol [46]; d) Transgenic mice over-expressing CYP2E1 showed an enhancement of hepatic injury following chronic ethanol administration [43, 47, 48]; e) in (HepG2) liver cells over-expressing CYP2E1, incubation with rising ethanol concentrations led to a linear increase of εdA levels in DNA, which was inhibited by small amounts of CMZ [49].

Liver biopsies from patient with varying degree of ALD severity were immunohistochemically analyzed for CYP2E1, 4-HNE, and εdA adducts. Again, we found a significant positive correlation for CYP2E1 vs. 4-HNE as well as for CYP2E1 vs. εdA [49].

In an ongoing study analysis of liver biopsies from about hundred ALD patients confirmed at a high level of significance these correlations and the association between hepatic fibrosis, CYP2E1 and εdA (Seitz, personal communication). These data strongly implicate CYP2E1 as an important mediator for etheno DNA adduct formation and this detrimental DNA damage may act as a driving force for ALD progression.

Since chronic ethanol consumption is also a risk factor for esophageal and colorectal cancer we also measured CYP2E1 and εdA in these tissues. In 37 patients with esophageal cancer esophageal biopsies adjacent to the tumor were analyzed and were compared to control biopsies from 12 non-alcohol drinkers [50]. In the esophageal mucosa a significant correlation between the quantity of alcohol intake and CYP2E1 induction as well as etheno-DNA adduct formation was found. Both etheno-adducts εdA and εdC correlated significantly with CYP2E1 [50], while control patients did not show CYP2E1.

CYP2E1 can also induced in the colorectal mucosa. In our study in heavy and light drinkers no difference in CYP2E1 and εdA levels was observed, possibly due

to dietary modulators that affect CYP2E1, LPO, and adduct production in situ. However, when the data of all patients (controls and alcoholics) were pooled, a significant correlation between CYP2E1 and εdA became apparent [51].

6.5 Etheno DNA Adducts in Animal Models of NASH with and Without Additional Alcohol Administration and in NASH Patients

Based solely on histomorphology NASH and ASH are very difficult to distinguish. In both ALD and NASH CYP2E1 was reported to be induced [39–41], this induction in NASH is less expressed than in ALD for reasons that we investigated.

Formation of εdA in a cohort of patients with NASH has clearly been established [38], but the etheno adducts did not correlated with CYP2E1. To explain this unexpected finding we assume that in NASH inflammation predominates rather than CYP2E1 induction, and etheno adducts are formed via ROS generated during inflammatory processes (Fig. 6.1) In this context it is noteworthy that in NASH patients a significant correlation was noted between CYP2E1 and hypoxemia and ß-hydoxybutyrate [52].

In a further study we investigated etheno-DNA adducts in 21 children and adolescents who were diagnosed with NASH with and without diabetes [53]. In 3 out of 21 children etheno DNA adducts were extremely high. Since alcohol consumption even at social levels increase the risk for hepatocellular cancer in NASH it would be important to monitor these children for HCC further in life.

In a series of animal experiments where NASH was induced either in genetically modified rodents or by feeding a high fat diet we further investigated the formation of etheno-DNA adducts. When obese Zucker-rats who are leptin deficient and insulin resistant received alcohol as Lieber-DeCarli-diets etheno-DNA adducts increased to a much higher degree in obese as compared to lean Zucker-rats and this increase was further enhanced when alcohol was administered. Etheno adduct formation was highly significant and paralleled by the level of hepatic CYP2E1 [49].

When Sprague-Dawley-rats received a Lieber-DeCarli-high-fat-diet with 71% energy from fat NASH was produced within 6 weeks. Afterwards these rats were continuously fed with high fat diet (55% total energy from fat) or high fat plus alcohol diet (55% energy from fat and 16% energy from ethanol) for another 4 weeks [54]. High fat diet alone increased hepatic inflammation and apoptosis as compared to a control diet, and nearly doubled the level of hepatic etheno-DNA adducts and of CYP2E1. The addition of ethanol did not significantly affect parameters associated with lipid peroxidation, inflammation and apoptosis, and no further increase in etheno-adducts and of CYP2E1 was noted [55] (Fig. 6.4).

A similar observation was made in a mouse model [56] where multiple binge-drinking with an ethanol intake of 2 g/kg body weight twice a week for 12 weeks increased etheno-DNA adducts in the liver only to a minor degree as compared to a

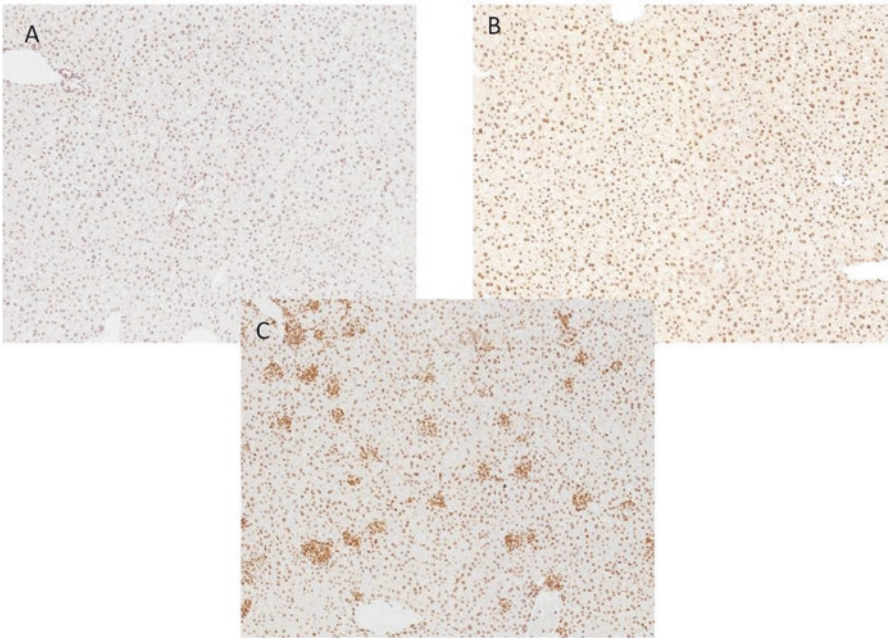

Fig. 6.4 Effect of binge drinking and high fat diet on hepatic etheno-DNA adducts (εdA). (**a**) single dose of ethanol (2 g/kg), (**b**) multiple binges (2 g/kg, 12 weeks, twice per week), (**c**) multiple binges (2 g/kg, 12 weeks, twice per week plus high fat diet (45% energy from fat). While multiple binges increase εdA moderately without reaching the level of statistical significance (**b**) as compared to a single dose of ethanol (**a**), multiple binges combined with a high fat diet significantly increase hepatic εdA which occur predominantly in clusters (**c**)

single binge of 6 g/kg body weight in an alcoholic steatosis model [56]. However, when multiple binges were combined with a high fat diet (45% of total calories from fat) a striking elevation of etheno DNA adducts was found. Interestingly, these etheno-DNA adducts occurred in clusters within the liver (Fig. 6.4).

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Chapter 7
Role of TGF-β in Alcohol-Induced Liver Disease

Wilma Jogunoori and Lopa Mishra

Abstract Over 90% of hepatocellular carcinoma (HCC) occurs against a background of chronic liver disease or cirrhosis induced from viral hepatitis to alcohol injury. One third of patients with cirrhosis will develop HCC during their lifetime, with a 3–5% annual incidence. However, little is known about the key mechanisms by which toxins mediate DNA damage in the liver. Recent studies support a central role for TGF-β signaling in conferring genomic stability yet the precise mechanism of action and the specific stages of tumor suppression remain unclear (Bornstein S, White R, Malkoski S, Oka M, Han G, Cleaver T, Reh D, Andersen P, Gross N, Olson S, Deng C, Lu SL, Wang XJ. J Clin Invest 119:3408–3419 (2009); Korc M. J Clin Invest 119:3208–3211 (2009); Glick A, Popescu N, Alexander V, Ueno H, Bottinger E, Yuspa SH. Proc Natl Acad Sci U S A 96:14949–14954 (1999)). Furthermore, it has recently been shown that *β2SP*$^{+/-}$ and *β2SP*$^{+/-}$*/Smad3*$^{+/-}$ mice phenocopy a hereditary human cancer syndrome, the Beckwith-Wiedemann syndrome (BWS), which has an 800 fold risk of cancers including HCC, hepatoblastoma, and a range of liver disorders. Identifying key biological pathways and mechanisms for suppressing alcohol-induced stem cell injury and HCC will be critical for enhancing patient care and the employment of new therapeutic approaches.

Keywords Hepatocellular · Carcinoma · Cirrhosis · TGF-β · Beckwith-Wiedemann syndrome

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7.1 Background and Significance

Currently, alcohol is consumed by nearly 75% of the population in the U.S.; worldwide, approximately 2.5 million people die each year from alcohol-related causes (WHO "Global Status Report on Alcohol and Health."). Nearly 20% of these deaths result from end-stage liver disease or cirrhosis. The complex detoxifying function of the liver renders it to phenomenal cellular and genotoxic stress that needs to be overcome to prevent cirrhosis and malignancy. The transforming growth factor β (TGF-β) signaling pathway is instrumental in mammalian development as well as in tumor suppression, through inhibition of proliferation and induction of apoptosis in multiple cell types. TGF-β has also been linked to detoxification and DNA repair mechanisms in the liver. Yet paradoxically TGF-β has a *Jekyll and Hyde* role in tumorigenesis—though TGF-β signaling deters the initiation of cancer, it also promotes tumor cell invasiveness and metastasis through modulating the immune system as well as the microenvironment. Molecular mechanisms and clinical relevance of the tumor suppressive effects of TGF-β are becoming increasingly evident. However, insight into the context dependent roles of this powerful pathway will be critical for the efficacious development of new therapeutics.

Detoxification and DNA repair are critical functions of the liver, and need to be tightly regulated to maintain homeostasis. However, we are only beginning to understand the mechanisms that occur when detoxification and DNA repair are disrupted through acute and chronic insults. Elucidating the links between TGF-β signaling and DNA repair could potentially lead to the identification and validation of new causative targets and signaling pathways underlying alcohol-induced liver injury and tumorigenesis that are urgently needed. Over 90% of HCC occurs against a background of chronic liver disease and cirrhosis from viral hepatitis to alcohol injury. Identifying key biological pathways and mechanisms for suppressing alcohol-induced injury and HCC could lead to new therapeutics—as new approaches have used PARP inhibitors for *BRCA1* mutant breast cancers.

7.2 Overview of TGF-β Signaling

TGF-β was first discovered in 1986 and has generated a vast number of studies with over 50,000 publications in part due to its multiple and often paradoxical functions. Signal transduction mediated by TGF-β related factors is a classic example of receptor serine/threonine kinase cell signaling. Simply, ligand binding initiates dimerization of the type II to the type I receptor which in turn activates Smad proteins and thus transcription (Fig. 7.1). Bone Morphogenic Protein (BMP) signaling is closely related to TGF-β, but each pathway differs in their ligands, Smad involvement, and in the regulation of developmental and cellular processes [12, 24, 31, 46]. Activins, Nodals, and TGF-βs are the ligands that initiate signal transduction in the TGF-β pathway through regulatory Smads (R-Smads), namely Smad2/3, Smad4 (co-smad)

or antagonize through inhibitory Smad6/7 [3, 18, 23, 30]. Ligand binding stimulates type II receptors (TGF-βRII, ACVR2A/B) to dimerize with type I receptors (ALK4/5/7) which phosphorylate the last two serine residues within a highly conserved SSXS motif at the carboxyl terminus of R-Smads [1, 32, 37, 38, 55]. Once phosphorylated, R-Smads associate with the common Smad, Smad4 [62] and mediate nuclear translocation of the heteromeric complex. In the nucleus, the Smad complexes mediate transcription through cooperative interactions with transcription factors to regulate developmental processes during embryogenesis including growth, differentiation, wound repair, cell polarity, and apoptosis. TGF-β has been shown to suppress tumor formation through genetic studies in murine knockout models of TGFβ type I and type II receptors, as well as the common mediator Smad4 [40, 45, 18, 56, 58]. However, persistent high levels of TGF-β promote malignancies and metastases. The multifaceted effects of TGF-β are due to the multi-level regulation at each step in the signaling cascade as well as the cellular context [11, 20, 28, 54].

7.3 Modulation of TGF-β Signal Transduction

As TGF-β signaling has been identified as a major signaling pathway throughout development and disease, much attention has been given to the key regulators of this pathway. In the cytoplasm, Smad function is modulated by adaptor proteins such as SARA, β2SP, filamin, and others [45, 57, 26]. The function of SARA is to recruit R-Smads to the type I receptor, to facilitate Smad phosphorylation and activation. The Smad adaptor β2SP, also phosphorylated by type I receptors, binds the activated R-Smad together with common Smad4 and translocates to the nucleus to activate transcription [45, 56, 26] (Fig. 7.1).

Smad binding is required to activate transcription of target genes, through interactions with sequence specific transcription factors and co-activators P300/CBP, despite the fact that Smads have a 100-fold lower affinity than the interacting high affinity DNA binding transcription factors. Through interactions with a variety of transcription factors, TGF-β signaling is able to promote transcriptional networks with high specificity. For example, the AP1 transcription factor is needed for TGF-β induced PAI-1 and collagenase expression [15, 27]. TGF-β mediated transactivation of cyclin-dependent kinase inhibitors p21 (Datto et al. 1995) and p15 involves the SP1 transcription factor [36]. The tumor suppressor protein TCS2 also interacts with Smad2 and Smad3 and potentiates the transcriptional regulation of p21 and p27, while repressing cyclin A expression and thus eliciting growth arrest [9].

TSC2 is an established downstream effector of the LKB1 tumor suppressor, and mediates the anti-proliferative effects of LKB1 in epithelial cells [2]. These findings represent an interesting point of convergence between two major tumor suppressor pathways, TGF-β and LKB1. In response to the Activin ligand, the FAST-1 transcription factor binds Smad2 and recruits Smad4. Upon nuclear translocation, these proteins become part of a larger complex known as the Activin Response Factor

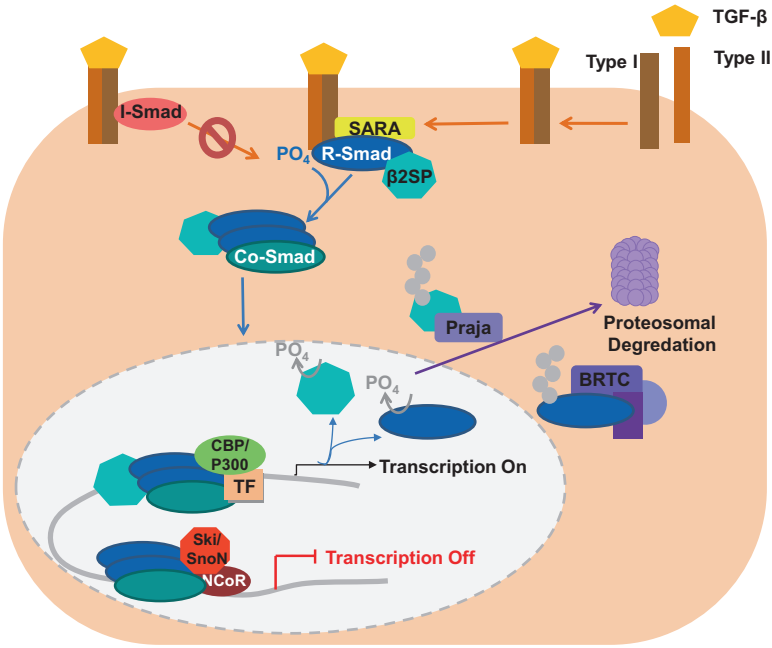

Fig. 7.1 Overview of TGF-β signaling. TGF-β ligand binds to Type II serine threonine kinase receptor and initiates the dimerization to Type I receptor. The activated receptor complex phosphorylates Regulatory Smads (R-Smads) which are recruited to the receptors with the help of adaptor proteins such as SARA and β2SP. Activated R-Smads then bind to the Common Smad (Co-Smad) and translocate to the nucleus where the Smad complex binds specific transcription factors (TF) and histone acetyltransferase P300/CBP to activate target genes transcription. This signaling cascade can be regulated by inhibitory Smads (I-Smads), Ski/SnoN and E3 ligase. I-Smads compete with R-Smads for receptor binding and halt the signaling cascade. Ski/SnoN blocks P300/CBP binding to the Smad complex, and recruits NCoR to silence transcription. De-phosphorylation of β2SP and R-Smads facilitates transport back to the cytosol. The E3 ubiquitin ligase such as SCF type E3 ubiquitin ligase complex BRTC targets R-Smads for proteosomal degradation, while Prajal targets both β2SP and R-Smads for degradation. Arrows indicate signal flow and are color coded: orange for ligand and receptor activation, blue for Smad activation, and purple for ubiquitin mediated protein stability. Ubiquitin is depicted as grey circles

(ARF); which is a critical regulator of early embryogenesis [12]. Interestingly, recent studies have shown that TGF-β signaling regulates the CTCF, an insulator protein that plays a critical role in chromatin structure, in a β2SP dependent manner [13]. Altogether these findings highlight the tumor suppressor functions of TGF-β signaling, and implicate this pathway as a guardian of genomic stability.

TGF-β signaling is negatively regulated by other proteins on many levels. Though inhibitory ligands and Smads play a role, there are other mediators further downstream. A classic example of this is negative regulation by Ski/SnoN, which pre-

vents P300/CBP binding and recruits the transcriptional co-repressor NCoR to gene promoters to instill gene repression [17]. Balance of the ubiquitin-proteosome pathway is also critical as proteins are targeted for degradation by various E3 ubiquitin ligases. Smad3 stability is mediated by a specific SCF ubiquitin ligase complex, BTRC, while β2SP is targeted by the RING-domain E3 ligase Praja [39].

7.4 Alcohol Induced TGF-β Signaling

As the detoxifying organ of the body, the liver is subjected to an immense amount of cytotoxic and genotoxic stress. This stress becomes magnified upon repeated exposure to alcohol, as well as in the context of hepatitis. Though different cell types within the liver respond to this stress in different ways, each cell type seems to have defense mechanisms in place to overcome acute exposure to alcohol—though these programs become deregulated with repeated or chronic insults.

Hepatocytes, or parenchymal cells, are the most abundant cell type in the liver. These cells facilitate many important metabolic functions including glycogen storage, as well as detoxification of ammonia and alcohol. In a mouse model of acute alcohol exposure, and to a lesser extent in chronic alcohol exposure, PAI-1 was required for protection against hepatic steatosis [41]. Given that TGF-β signaling positively regulates the expression of PAI-1, TGF-β signaling may have a protective effect of hepatocytes in response to alcohol [15, 27]. Through chronic exposure to alcohol, TGF-β signaling has an adverse effect on hepatocyte metabolism. In the livers from mice treated with chronic ethanol insults, TGF-β activation led to an increase in stress response and fibrotic transcriptional programs.

Interestingly, TGF-β induction led to a decreased expression of alcohol dehydrogenase I (ADH1) which subverted these metabolites towards other pathways, and promoted steatosis of the liver [14].

Stellate cells of the liver have opposing reactions to alcohol induced stress depending on the exposure level. In the context of acute alcohol exposure, TGF-β signaling promotes transcriptional programs of wound healing. However, upon chronic exposure of alcohol, stellate cells no longer provide TGF-β induced wound healing; instead, these cells illicit a pro-fibrotic response through TGF-β mediated activation of p38-MAPK signaling [39, 19].

The liver is also home to a variety of immune cells including the resident macrophages called Kupffer cells, as well as lymphocytes including natural killer cells (NK) and dendritic cells (DC). Upon acute exposure to alcoholic stress the immune cells in the liver collectively balance the secretion of pro-inflammatory cytokines, such as tumor necrosis factors (TNFs) and interferons (INFs), with anti-inflammatory responses through interleukins such as IL-10 [50]. Upon chronic alcoholic exposure, however, TGF-β mediates the immune cells to change their response and favor the secretion of pro-inflammatory cytokines.

7.5 Defective TGF-β Signaling in Cirrhosis and Hepatocellular Carcinoma

TGF-β exerts bi-modal effects acting in a tumor suppressive or oncogenic fashion depending on the stage of the disease. Upon chronic alcohol exposure, this signaling pathway promotes cirrhosis through encouraging steatosis, fibrosis, and inflammation. In early oncogenesis, especially in hepatocellular carcinoma (HCC), TGF- β mainly elicits tumor suppressive actions, while in later stages of HCC it promotes metastases [39]. In a cirrhotic and pre-malignant condition, the persistent exposure to alcohol reinforces a pro-inflammatory feedback loop in Kupffer cells and CD133+/CD49f+ tumor initiating cells (TICs). Alcohol induces serum endotoxin levels which stimulate TLR4 on both Kupffer cells and TICs to promote inflammation, in the absence of TGF-β signaling. Interestingly, pluripotency factors such as OCT4 and NANOG are required for this mechanism. The NANOG transcription factor promotes IGF2BP3, which in turn promotes YAP phosphorylation, and together IGF2BP3 and YAP inhibit the phosphorylation and subsequent nuclear translocation of Smad3 [60, 39].

These data coincide with the role of insulin-like growth factor (IGF) signaling in tumorigenesis. IGF signaling plays a major role in mammalian organismal growth, and activation of IGF2 and IGF1R have been observed in various epithelial cancers [35, 25, 52].

Moreover cells lacking IGF1R are unable to undergo oncogenic transformation. Thus, signaling through the IGF pathway appears to be a key oncogenic component, and has been implicated in resistance to certain anti-cancer regimes [6, 44, 51, 53]. In the context of defective TGF-β signaling, from either $\beta 2SP^{+/-}$ or $\beta 2SP^{+/-}/Smad3^{+/-}$ tissues and tumors, IGF2 expression markedly increased. In the malignant state, the IGF pathway presumably functions through activation of the PI3K/Ras/MAPK/ERK pathways to promote proliferation and induce anti-apoptotic effects—both of which are required for tumor growth [13, 61]. Altogether, the dampening of TGF-β signaling and the activation of IGF signaling is critical for the initiation of HCC. Furthermore, these findings substantiate the development of small molecule inhibitors for the IGF pathway and highlight this approach as a promising avenue in cancer therapy.

However, the role of TGF-β is reversed in metastatic HCC. Levels of this cytokine are low in non-metastatic HCC, however the levels drastically increase in patients with aggressive metastatic disease. A hallmark mechanism of metastases, from solid tumors, is epithelial to mesenchymal transition (EMT) in which cells are more prone to migrate through loss of cell-to-cell adhesions. In metastatic HCC, TGF-β promotes the transcription of a key regulator of EMT called SNAI1 [21]. Given its extreme and context dependent roles in the initiation and progression of cancer, caution should be taken when considering direct pharmacological inhibition of TGF-β signaling.

7.6 The Role of TGF-β in Genomic Stability and Stem Cell Disorder Beckwith-Wiedemann Syndrome

It has been proposed that TGF-β functions as an extracellular sensor of damage caused by UV and ionizing radiation [5, 4]. Multiple studies have demonstrated that TGF-β plays a role in the interstrand crosslink (ICL) repair system—more specifically the Fanconi anemia/Breast cancer 1 (Fanc/Brca1) DNA repair pathway. Smad4 conditional knockout mice have demonstrated that Smad4 regulates the expression of Fanconi related genes [10, 43]. Moreover, β2SP associates with Fanconi group proteins and localizes to interstand crosslinks (ICL)—confirming that TGF-β signaling is sensitive to DNA damage and promotes DNA repair through ICL repair systems [42, 33]. One of the most formidable of post replication DNA lesions is the replication fork lesion—a barrier to chromosome duplication—which leads to mitotic catastrophe, complex chromosome rearrangements, and cell death. These lesions are managed by ICL repair mechanisms such as Fanc/Brca1 to prevent replication fork progression [16]. This complex repair mechanism has central components including the Fanconi complex, an E3 ligase, and at least 4 other factors. The central complex in this pathway is formed by the FAD2 (FANC D2) and FAI (FANCI) proteins forming the ID complex that are phosphorylated by the ATR kinase (Fig. 7.2). The failed response to toxins such as alcohol was observed in null models of Fanconi genes, and led to the production of reactive aldehydes and adducts that directly damaged DNA, which ultimately led to the development of cancer [34]. Given that TGF-β signaling promotes the expression of and interacts with members of this repair pathway, it is clear to see how defective TGF-β signaling can lead to genomic instability and oncogenesis.

Recently, a dramatic finding has implicated the TGF-β signaling pathway in the development of a stem cell disorder called Beckwith-Wiedemann Syndrome (BWS). This syndrome is an overgrowth disorder associated with an increased risk of neoplasm development, estimated between 4% and 21%, as initially described by Beckwith in 1963 [7,8]. However, no causal mutation has been linked with the development of BWS. Through genetically engineered mouse models, it has been demonstrated that *β2SP*$^{+/-}$ and *β2SP*$^{+/-}$*/Smad3*$^{+/-}$ mice develop a phenotype that is nearly identical to BWS, as mice develop visceromegaly and multiple tumors including metastatic pancreatic cancer and hepatocellular carcinoma, among others [13]. Considering that BWS has an incidence of 1 per 6-10,000 births in the US, and may be rising 3-4 fold in children from *in vitro* conception in some countries, understanding the precise involvement of TGF-β signaling in this disorder may provide opportunities for intervention [48, 59].

When examined further, cell lines made from *β2SP*$^{+/-}$ and *β2SP*$^{+/-}$*/Smad3*$^{+/-}$ mice showed an upregulation of pluripotency markers such as *SOX2* and *CD34*, similar to human BWS cell lines. Interestingly, all of these cell lines also harbor overexpression of *IGF2* and a downregulation of *H19*. This finding further validated the *β2SP*$^{+/-}$ and *β2SP*$^{+/-}$*/Smad3*$^{+/-}$ mice as models of BWS. A hallmark of BWS is loss of imprinting (LOI) on chromosome 11 at the 11p15 locus, encoding *BWR1C*,

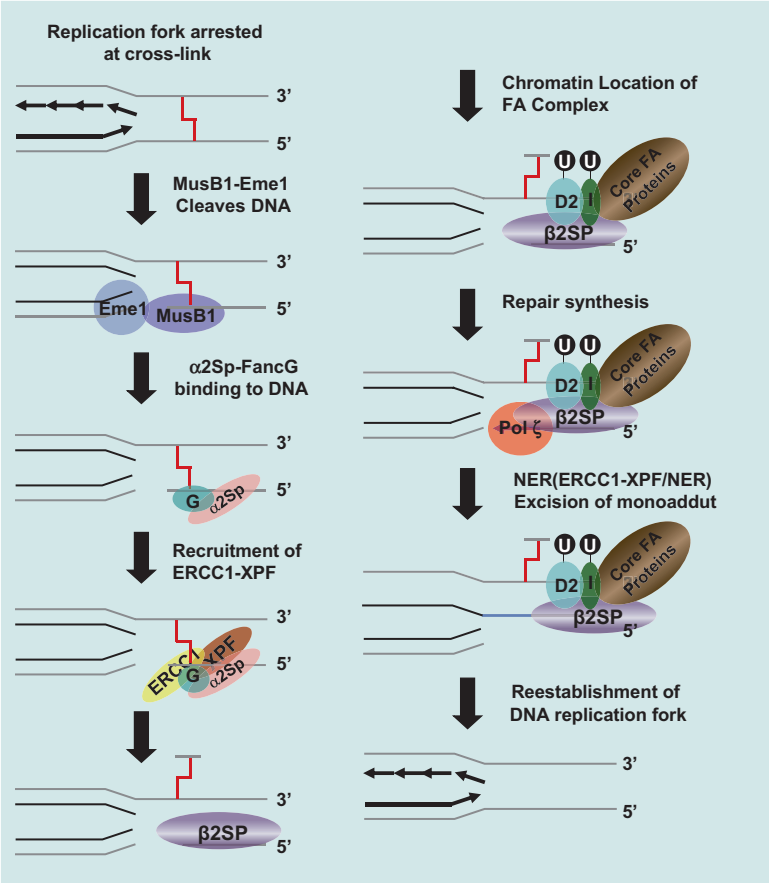

Fig. 7.2 Model for Interstrand Cross-Link (ICL) repair at an arrested replication fork with a double stranded break (DSB). Events include unhooking of the cross link (red), potential recruitment of FANC complex by β2SP, and homologous recombination machinery, followed by DSB repair and re-establishment of the replication fork

CDKN1C, and *IGF2*, among others. Intriguingly, defective TGF-β signaling may play a causative role in the LOI of *IGF2*, considering the previously established link in HCC. Therefore understanding the epigenetic deregulation that occurs simultaneous to the LOI is critical for a better understanding of the molecular etiology of BWS. Recent studies implicate CTCF as a link between these data. β2SP and Smad3 are required for CTCF stability in the liver, and these proteins directly interact in HepG2 cells upon exogenous stimulation by TGF-β [13]. Moreover, CTCF—an evolutionarily conserved 11-zinc finger, DNA-binding nuclear phosphoprotein—is involved in multiple aspects of normal gene regulation that include chromatin insulation, transcriptional repression and activation, gene silencing, and regulation of imprinted sites [47, 49]. In the context of β2SP and Smad3, CTCF transcriptionally

repressed the *TERT* locus. In BWS and in *β2SP*$^{+/-}$*/Smad3*$^{+/-}$ mice, the dysfunction and deregulation of the β2SP/Smad3/CTCF complex caused an increase in *TERT* expression and enhanced the tumorigenesis of CD133+ tumor initiating cells. It is possible that BWS may result directly from β2SP mutations, and that the *β2SP*$^{+/-}$, *β2SP*$^{+/-}$*/Smad3*$^{+/-}$ mutant mice represent a novel pathway for BWS. It is plausible that imprinting plays a significant role in the observed phenotype and that the tumors arise due to deregulation of TGF-β mediated pathways including IGF signaling, more specifically IGF2.

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Chapter 8
NANOG-Dependent Metabolic Reprogramming and Symmetric Division in Tumor-Initiating Stem-like Cells

Keigo Machida

Abstract Alcohol abuse synergistically heightens the development of the third most deadliest cancer hepatocellular carcinoma (HCC) in patients infected with hepatitis C virus (HCV). Ectopically expressed TLR4 promotes liver tumorigenesis in alcohol-fed HCV *Ns5a* or *Core* transgenic mice. CD133+/CD49f + tumor-initiating stem cell-like cells (TICs) isolated from these models are tumorigenic have p53 degradation via phosphorylation of the protective protein NUMB and its dissociation from p53 by the oncoprotein TBC1D15. Nutrient deprivation reduces overexpressed TBC1D15 in TICs via autophagy-mediated degradation, suggesting a possible role of this oncoprotein in linking metabolic reprogramming and self-renewal.

Keywords HCC · Cancer stem cells · Tumor-intiating stem-like cells (TICs) · NUMB

8.1 Introduction

Major risk factors for HCC are HCV, HBV, alcoholism, and obesity [12, 31]. Alcoholic liver disease (ALD) and viral hepatitis (HBV and HCV) are associated with development of hepatocellular carcinoma (HCC) [30] as more than 170 million people are infected with HCV worldwide [30, 31, 45]. HCV proteins (Nucleocapside Core and others) are linked to transformation through overproduction of reactive oxygen species which may cause mitochondrial or nuclear DNA damage [19, 28, 31]. The core protein also inhibits microsomal triglyceride transfer protein activity and VLDL secretion [32], which contributes the genesis of fatty liver. The core also induces insulin resistance in mice and cell lines, and this effect may be mediated by degradation of insulin receptor substrates (IRS) 1 and 2 via up regulation of SOCS3

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[16] in a manner dependent on PA28γ 73, or via IRS serine phosphorylation [5]. HCV-induced mechanisms promotes HCC risk with non-alcoholic fatty liver disease (NAFLD) (9). HCV/HBV infection, ALD, and NAFLD share common pathophysiological events such as oxidant stress, organelle stress, and metabolic dysregulation which may contribute to their oncogenic activities.

Refractoriness to chemotherapy after HCC treatment is challenging via genesis of tumor-initiating stem cell-like cells (TICs) or so-called cancer stem cells (CSCs). Stem cells have three major characteristics, self-renewal, asymmetric division (clonality), and plasticity. Forty percent of HCC are assumed to have clonality and to originate from progenitor/stem cells [1, 34, 39, 48]. CD133+/CD49f + cells in liver tumors correlate with tumorigenesity and the expression of "stemness" genes, such as Wnt/β-catenin, Notch, Hedgehog/SMO, and Oct3/4 [6, 9, 40]. Indeed, CD133+/CD49f + HCC TICs are chemoresistant [35], survive during an initial therapy. Although an encouraging therapeutic response may be seen, survived TICs eventually establish a clonal expansion and tumor recurrence. This chemoresistance may be caused by the plasticity of TICs with dysregulated signaling and gene expression. Several oncogenic signaling pathways are activated in HCC or TICs, including PI3K/AKT [24], signal transducer and activator of transcription 3 (STAT3) [43, 46], and hedgehog [37, 38] while defective tumor suppressor transforming growth factor-beta (TGF-β) pathway is also implicated [18, 29]. Another pivotal mechanism is asymmetric division of TICs producing dormant daughter cells which are less sensitive to chemotherapeutic drugs.

8.2 Synergistic Risk Factors for Alcohol-Associated HCCs

Co-existence of alcohol abuse or obesity, increases the HCV risk of developing HCC by additional eight fold, culminating to an overall 45–55 fold increase in the risk as compared to normal subjects (10,11). As alcohol and obesity continue to dominate as leading life-style factors for disease burdens around the world (12), heightened HCC incidence caused by synergistic interactions of these factors with hepatitis viruses, represents the most predictable and devastating global health issue. Compelling evidence identifies a synergism between obesity/alcohol and HCV infection with the associated risk of developing HCC [47]. The risk of HCC increases from 8–12 to 48–54 by co-morbidities such as alcoholism or obesity [47]. Obesity and alcoholism increase gut permeability leading to endotoxemia, which in turn activates Toll-like receptor 4 (TLR4) in the liver with production of cytokines and an inflammatory response. This leads to subsequent development of obesity/alcohol-related liver disease [13]. Therefore, to develop better therapeutics, the underlying molecular mechanisms regulating obesity/alcohol/HCV-induced hepatocarcinogenesis should be elucidateds.

8.3 Genesis of TICs Induced by Alcohol Exposures

Liver-specific expression of the HCV NS5A protein in mice fed alcohol for 12 months develop liver tumors in a TLR4-dependent manner [11]. Circulating endotoxin binds CD14-TLR4 complex, activates hepatocytes/hepatoblasts and induces the stem cell marker NANOG. This process generates TLR4/NANOG-dependent, chemoresistant tumor-initiating stem-like cells (TICs; $CD133^{+}$), which can induce HCC in mice [11].

TICs are rare, highly malignant cells that are present in diverse tumor types and play a central role in tumorigenesis, malignant progression, and resistance to chemotherapy [25, 35]. Sorafenib, a multi-kinase inhibitor, is the most commonly used monotherapy agent for the treatment of HCC; however, resistance to sorafenib eventually occurs in patients [41]. We recently reported that treatment with sorafenib made TICs more susceptible to tumor growth retardation, with a decrease in tumor size by ~55% when combined with knockdown of NANOG-inducible proto-oncogenes (including YAP1, which induces antioxidant gene programs) [11]. However, the underlying mechanism of chemoresistance and self-renewal of TICs remains incompletly understood.

8.4 TLR4/NANOG-Dependent TICs Give Rise to Tumors

Mouse-derived tumors contain double-positive cells for NANOG and CD133 or CD49f (24). TLR4 silencing reduces heightened expression of stemness genes and cell proliferation [10]. CD133+/CD49f + cells are TLR4/NANOG-dependent TICs and that *Tlr4* is a putative proto-oncogene involved in the genesis/maintenance of TICs and liver tumor in HCV Tg models. Hepatoblastic HCC subtype with poor prognosis has a gene expression profile with markers of hepatic oval cells [3, 8, 20, 44]. HCC often recurs after chemotherapy due presumably to the presence of chemo-resistant TICs [33].

8.5 Metabolic Reprogramming and TIC Self-Renewal

Toll-like receptor 4 (TLR4) signaling phosphorylates E2F1 to transactivate NANOG. Down-regulation of *Nanog* reduces tumor progression. NANOG ChIP-seq identified genes associated with NANOG-dependent mitochondrial metabolic pathways to maintain tumor-initiating stem-like cells (TICs). The causal roles of NANOG in mitochondrial metabolic reprogramming occurred through the inhibition of oxidative phosphorylation (OXPHOS) with decreased production of

mitochondrial ROS and activation of fatty acid oxidation (FAO), which was required for self-renewal and drug resistance [10]. Restoration of OXPHOS activity and inhibition of FAO rendered TICs susceptible to a standard care chemotherapy drug, sorafenib [10].

Complementary NANOG ChIP-seq and metabolomics studies of TICs demonstrated that NANOG induced by TLR4 suppressed mitochondrial OXPHOS and activated FAO, thus inhibiting OCR and ROS production. This conferred a tumor chemoresistant state which could be abrogated by NANOG-targeted gene silencing. Our findings demonstrated a NANOG-dependent downstream effect on mitochondrial function in TICs that contributed to the mechanism of chemotherapy resistance [10]. These metabolic reprogramming promoted self-renewal/oncogenesis, and explained how NANOG activation inhibited therapy-mediated apoptosis by quenching ROS production. Restoration of OXPHOS and activation of decreased FAO reduces tumorigenic capacity of TICs and increases susceptibility to chemotherapy [10].

As TICs rely on active FAO for their maintenance and function, FAO inhibitor suppresses self-renewal of leukemiainitiating cells (LICs) [36]. We experimentally reversed the effects of FAO gene silencing and restored the original TIC phenotype by overexpression of FAO genes. Thus the fate of stem cells is metabolically switched by FAO [14]. Potential mechanisms by which elevation of FAO maintains self-renewal ability include: (i) shunting of long-chain FA away from lipid and cell membrane synthesis; (ii) downregulation of ROS through production of NADPH to avoid loss of TICs; and (iii) reduction of metabolic resistance to chemotherapy. By these criteria, NANOG function could be construed to serve as a gatekeeper for FAO activity.

8.6 Cell Fate Determinant NUMB and Oncogenesis in TICs

Stem cell populations are maintained through self-renewing divisions in which one daughter cell commits to a particular fate while the other retains the multipotent characteristics of its parent. Tumor-initiating cells (TICs) contribute to oncogenesis and progression to treatment-refractory metastatic disease [22, 23, 42] with the heightened expression and activation of a pluripotency-associated transcription factor (TF) network [15]. The p53 tumor suppressor regulates pluripotency and stem cell division. Genetic deletion or shRNA-mediated depletion of p53 enhances cellular reprogramming to the pluripotent state [4, 17, 27] and p53 can directly repress the expression of pluripotency-associated TFs [21]. p53 is also required to maintain asymmetry and cell polarity in proliferating stem cells and interacts directly with the NUMB protein. A polarity determinant NUMB is distributed asymmetrically in dividing stem cells and is segregated into the daughter cell which undergoes differentiation. The association with a tumor suppressor Numb stabilizes p53 [7, 26]. Regulation of the assembly or stability of the Numb-p53 complex mediates TIC-derived oncogenesis. NUNB-associated oncoproteins MDM2 E3 ubiquitin ligase

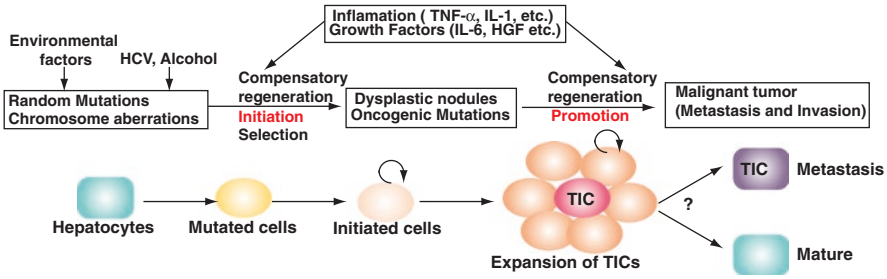

Fig. 8.1 Tumor initiation and genesis of TIC through environmental factors and virus infection

destabilize the Numb-p53 complex and promote proteolysis of p53 [2]. The NUMB, a tumor suppressor, in conjunctions with another tumor suppressor protein p53, preserves this property and acts as a barrier against deregulated expansion of Tumor-associated stem cells. In this context, NUMB-p53 interaction plays a crucial role to maintain the proper homeostasis of both stem cells, as well as differentiated cells. As the molecular mechanism governing the assembly and stability of the NUMB-p53 interaction/complex are poorly understood, we tried to identify the molecule/s govern this process. Using cancer cell lines, tumor-initiating cells (TICs) of liver, the mouse model and clinical samples, we identified that phosphorylations of NUMB destabilize p53 and promotes self-renewal of TICs by pluripotency-associated transcription factor NANOG dependent manner. NANOG phosphorylates NUMB via aPKCζ, through the direct induction of Aurora A kinase (AURKA) and the repression of an aPKCζ inhibitor, LGL-2. Phosphorylation of NUMB by aPKCζ destabilizes the NUMB-p53 interaction, p53 proteolysis and to deregulate self-renewal in TICs Fig. 8.1.

8.7 Conclusions and Discussions

We successfully isolated CD133+/CD49f + TICs which activate a unique TLR4-NANOG pathway as an integral component for their self-renewal and tumorigenic activities. These TICs are also identified in HCC sections of alcoholic HCV patients by immunostaining and isolated from such patients to validate induction of TLR4-dependent stemness genes and transformation. Based on this renewed concept, our studies have offered two novel insights into the molecular mechanisms of NANOG-mediated p53 degradation by disengagement from the protective NUMB protein via TBC1D15 interaction. Posttranslational modification of NUMB by NANOG-AURKA-aPKCζ pathway is an important event in TICs self-renewal and tumorigenesis. Hence, our work identifies the NANOG-NUMB-p53 signaling axis is an important regulatory pathway for TICS event in TICs self-renewal and liver tumorigenesis and suggest a therapeutic strategy by targeting NUMB-phosphorylation. However, further in depth in vivo and clinical studies are warranted to verify this

suggestion. These studies are now exploring potential mechanistic connections to metabolic programming known to occur in cancer cells and TICs in promoting and maintaining “stem cell fate” via molecular, genetic, and epigenetic mechanisms.

NANOG maintains chemotherapy resistance of TICs involving not only the direct activation of self-renewal via stemness genes, but also the subsequent metabolic reprogramming in these cells leading to amplification of TIC oncogenic activity and their overall survival. Our data showed that NANOG reprogramming of mitochondrial metabolism was indeed responsible for human TIC oncogenicity and chemoresistance. The metabolic bases of altered cell functions and cell fate in TICs define potentially new approaches for chemo-sensitization and elimination of TICs for more efficacious HCC therapies. These studies have led to a paradigm shift in our understanding the underlying basis of alcohol/HCV-associated cancer, thus facilitating future development of new personalized treatment strategies targeted towards NANOG+ TICs arising from obesity, alcohol, or HCV-related HCC. The studies provide insights into the mechanisms of NANOG-mediated generation of TICs, tumorigenesis and chemo-resistance due to metabolic reprograming of mitochondrial functions.

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Chapter 9
Diet Supplementation with Soy Protein Isolate, but Not the Isoflavone Genistein, Protects Against Alcohol-Induced Tumor Progression in DEN-Treated Male Mice

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Abstract Diethylnitrosamine-treated male mice were assigned to 4 groups: a casein-based 35% high fat ethanol liquid diet (EtOH), an EtOH diet made with soy protein isolate protein (EtOH/SOY), an EtOH liquid diet supplemented with genistein (EtOH/GEN) and a chow group. EtOH feeding, final concentration 5% (v/v), continued for 16 wks. EtOH increased incidence and multiplicity of basophilic lesions and adenomas compared to the chow group, ($p < 0.05$). The EtOH/SOY group had reduced adenoma progression when compared to the EtOH and EtOH/GEN group, ($p < 0.05$). Genistein supplementation had no protective effect. Soy feeding significantly reduced serum ALT concentrations ($p < 0.05$), decreased hepatic TNFα and CD-14 expression and decreased nuclear accumulation of NFκB protein in EtOH/SOY-treated mice compared to the EtOH group ($p < 0.05$). With respect to ceramides, high resolution MALDI-FTICR Imaging mass spectrometry revealed changes in the accumulation of long acyl chain ceramide species, in particular C18, in the EtOH group when compared to the EtOH/SOY group. Additionally, expression of acid ceramidase and sphingosine kinase 1 which degrade ceramide into sphingosine and convert sphingosine to sphingosine-1-phosphate

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(S1P) respectively and expression of S1P receptors S1PR2 and S1PR3 were all upregulated by EtOH and suppressed in the EtOH/SOY group, $p < 0.05$. EtOH feeding also increased hepatocyte proliferation and mRNA expression of β-catenin targets, including cyclin D1, MMP7 and glutamine synthase, which were reduced in the EtOH/SOY group, $p < 0.05$. These findings suggest that soy prevents tumorigenesis by reducing inflammation and by reducing hepatocyte proliferation through inhibition of EtOH-mediated β-catenin signaling. These mechanisms may involve blockade of sphingolipid signaling.

Keywords Ethanol · Hepatocarcinogenesis · Diethylnitrosamine · Soy · Genistein · Sphingosine · β-Catenin

9.1 Introduction

Alcohol-induced liver cancer occurs via several different interacting mechanisms at the level of both initiation and promotion [1, 2]. Formation of acetaldehyde and reactive oxygen species results in DNA damage as the result of ethanol (EtOH) metabolism by alcohol dehydrogenase and CYP2E1, and reduced DNA methylation as a result of disruption of one carbon metabolism may result in tumor initiation [2]. In addition, there is data demonstrating that EtOH can act as a tumor promoter [3, 4]. Our laboratory and others have shown that EtOH stimulates hepatocyte proliferation in rodent models coincident with development of liver injury and depletion of hepatic retinoid stores [4]. Treatment with retinoic acid can reverse the increase in hepatocyte proliferation after EtOH exposure [5]. Down regulation of retinoic acid receptor (RAR) signaling by use of a dominant negative has also been shown to increase hepatocyte proliferation and liver tumor promotion as a result of increased Wnt-β-catenin signaling [6]. Recently, we have developed a mouse model of EtOH-induced liver tumor promotion in which, in combination with tumor initiation during early development by the nitrosamine chemical carcinogen diethylnitrosamine (DEN). In this model, EtOH consumption during adulthood results in increased multiplicity of liver adenomas [4, 7]. We have shown that increased tumor promotion was associated with necroinflammatory injury, fibrosis, retinoid depletion and increased β-catenin signaling in both hepatocytes and the tumors in this model [4, 7]. Clinically, a significant percentage of liver tumors from alcoholics have also been shown to be β-catenin positive [8]. In many of these cases, mutations have been found in β-catenin, the phosphorylation site of GSK3β or in Axin resulting in β-catenin stabilization [9]. However, we did not observe β-catenin mutations in the mouse DEN-EtOH model [4, 7]. Transgenic mice expressing mutations in the β-catenin pathway do not have increased tumor initiation suggesting, consistent with our EtOH model, that increased Wnt-β-catenin signaling contributes to tumor promotion [8].

If this hypothesis is correct, then compounds which inhibit β-catenin signaling should be tumor protective in the mouse DEN-EtOH model. In this regard, there is

ample epidemiological evidence and experimental data to suggest that dietary factors found in soy foods such as soy protein isolate (SOY) are cancer protective in multiple tissues. Meta-analyses have demonstrated reductions in risk of mammary, prostate and colon cancer in soy consumers [10]. In addition, animal studies of chemical carcinogenesis have shown protection against DMBA- and NMU-induced mammary tumors and against AOM-induced colon tumors after consumption of SOY [11–13]. Cancer protective effects of SOY have been ascribed to the presence of the isoflavone genistein in soy foods [14] working via several pathways including inhibition of cellular proliferation; induction of apoptosis; inhibition of angiogenesis and through anti-oxidant effects [14]. Genistein and SOY have also been suggested to interfere with Wnt-β-catenin signaling in a rat model of AOM-induced colon cancer coincident with significant decreases in formation of aberrant crypts [15]. In addition, in cell culture studies, genistein has been shown to inhibit various components of the Wnt-β-catenin signaling pathway [16–18] and has been shown to induce apoptosis in hepatocellular carcinoma cell lines [19]. The present study was designed to determine if feeding SOY or pure genistein at concentrations found in SOY diets are protective against EtOH-induced liver tumorigenesis in the mouse DEN-ETOH model and if protection was observed, it was associated with inhibition of β-catenin activation.

9.2 Materials and Methods

9.2.1 In Vivo Mouse Model of DEN-EtOH Liver Tumor Promotion

DEN-treated and saline-treated male C57Bl6 mice received EtOH-containing LieberDe Carli diets for 16 wks as previously described [29]. Briefly, DEN-injected mice (PND 13) were randomly assigned to four weight-matched diet groups: a chow diet (n = 10, chow), an EtOH-containing liquid diet (n = 21, EtOH) and an EtOH-containing liquid diet containing soy protein isolate (n = 23, EtOH/SOY), and an EtOH-containing liquid diet containing genistein, 250 mg/kg diet, a level comparable to the concentration of genistein in SOY (n = 24, EtOH/GEN). All groups had access to water *ad libitum*. Liquid diets were formulated according to the LiebeDeCarli diet of 35% of energy from fat, 18% from protein, and 47% from carbohydrates (Dyets, Inc., Bethlehem, PA). EtOH was added to the Lieber-DeCarli liquid diet slowly by substituting EtOH for carbohydrate calories in a stepwise manner until 28% total calories were reached as previously described [4]. This dose constitutes a final EtOH concentration of 5.0% (v/v). Additionally, saline-injected mice were randomized into three liquid diet groups, a chow diet (n = 5), an EtOH (n = 10), an EtOH/SOY (n = 10) for 16 wks [29].

9.2.2 Tumor Pathology

For each DEN-treated mouse, formalin fixed lobes were embedded in paraffin, sectioned at 4 μm, stained with H&E, and examined under a light microscope and scored in a blinded manner by a veterinary pathologist (L.H). Within each lobe, lesions were counted at 40x magnification. Tumors were defined as follows – adenomas, a compressive lesion of any size without evidence of invasion or other criteria of malignancy; hepatocellular carcinoma, a compressive and invasive lesion with criteria of malignancy [4].

9.2.3 Biochemical Analysis of Liver Injury and Inflammation

Liver necrosis was assessed by measurement of serum alanine amino transferase (ALT) activity as described previously [4]. Kupffer cell activation (CD14 mRNA expression) and inflammation (expression of cytokine TNFα and IL-6 mRNA and the chemokine CXCL2 mRNA) were measured by real time RT-PCR analysis of expression of individual cDNA samples prepared from each group using SYBR green and an ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA). Primer sequences are given in Table 9.1. Gene expression was normalized against 18S rRNA. In addition, nuclear fraction and cytosolic fractions were isolated from livers using NE-PER Nuclear and Cytoplsmic Extraction kit (Thermo Fisher Scientific) as per manufacturer's instructions. Nuclear proteins were separated by SDS-PAGE and Western blotted with antibodies against the p65 subunit of NFκB (Cell Signaling, Danvers, MA). Protein loading was corrected for by staining for total protein with 0.1% amido black.

Table 9.1 Real-Time RT-PCR primer sequences

Gene	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
TNFα	GACGTGGAACTGGCAGAAGAG	GCCACAAGCAGGAATGAGAAG
IL-6	CTTCACAAGTCGGAGGCTTAAT	GCAAGTGCATCATCGTTGTTC
CXCL-2	TAAGCACCGAGGAGAGTAGAA	GTCCAAGGGTTACTCACAACA
CD14	CTAAGTATTGCCCAAGCACACTCA	CCCAACTCAGGGTTGTCAGACA
CyclinDl	TGCTGCAAATGCAACTGCTTCTTG	AAGGTCTGTGCATGTTTGCGGATG
GluS	TATTCCTCGTGCCCAGTTAATC	AAGAAAGGGTTGGTGTGTAGAG
MMP-7	GACTTGCCTCGGTTCTTAGTAG3	CCC TTGCGAAGCCAATTATG
SPTLC2	CAGGAGCGTTCTGATCTTACAG	CCGGACACGATGTTGTAGTT
CerSl	GCCTGACATTCCGTACTACTTC	GTCTTCCAGTTCACGCATCT
SPKH1	GGTACGAGCAGGTGACTAATG	GGACAGACTGAGCACAGAATAG
ASAH1	GTCCTCAACAAGCTGACTGTAT	CTATACAAGGGTCTGGGCAATC
S1PR1	TTCACTCTGCTCCTGCTTTC	CTGGCCTTGGAGATGTTCTT
S1PR2	CAACGGAGGCACTGACTAAT	TGGCAAATGTCTAGCCCTAAG
S1PR3	GGGAGGCGTGATGTAGTTATTT	CAGAGGTGTCTTCTACGCATTT

9.2.4 Analysis of Ceramide-Sphingosine Signaling

Ceramide species were detected in the livers of saline-treated mice receiving an EtOH or EtOH/SOY diet by MALDI-FTICR imaging mass spectrometry as previously described [21, 29]. MALDI-IMS analysis was performed using a Bruker Solarix 7 T FTICR mass spectrometer, equipped with a SmartBeam II laser operating at 1000 Hz, collecting spectra across the entire tissue in positive ion mode between (*m*/*z* 200–2000). A laser spot size of 25 μm, and a raster width of 200 μm for general profiling or 75 μm for high resolution images was empolyed collecting 800 shots per pixel. Data was reduced to .98 ICR reduction and loaded into FlexImaging 4.0 software (Bruker Daltonics) for data analysis, and generation of lipid images of interest. Within FlexImaging, all data was normalized using root mean square and intensities were thresholded appropriately. Lipid species were assigned by mass accuracy, both to an internal ceramide database and to an external database Lipid Maps. mRNA expression of enzymes and receptors involved in ceramide-sphingosine signaling were quantitated by real time RT-PCR analysis of expression of individual cDNA samples prepared from each group using SYBR green and an ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA). Primer sequences are given in Table 9.1. Gene expression was normalized against the housekeeping gene GAPDH mRNA. In addition, liver microsomes were prepared as previously described [20] and used for Western blot analysis with an antibody against sphingosine kinase H1 (Cell Signaling, Danvers, MA). Protein loading was corrected for by staining total protein with 0.1% amido black.

9.2.5 Hepatocyte Proliferation and β-Catenin Signaling

Hepatocyte proliferation was measured by histochemical analysis of PCNA staining as described previously [22]. Nuclear expression of β-Catenin protein was measured by Western blot [4]. In addition, mRNA expression of known downstream β-Catenin target genes cyclin D1, glutamine synthase (GluS) and matrix metalloproteinase 7 (MMP7) were measured by real time RT-PCR as described above and expressed relative to GAPDH mRNA.

9.2.6 Data and Statistical Analysis

Data are presented as mean ± SEM. For ALT, gene and protein expression data multiple group comparisons were made by One Way ANOVA or ANOVA of Ranks followed by Student-Neuman-Keuls post-hoc analysis. Adenoma incidence was determined using Fisher's Exact Test. Multiplicity was determined One-Way

ANOVA followed by Mann-Whitney U rank-sum test for post hoc comparisons. Statistical analysis was performed using Sigma Plot 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 $P < 0.05$.

9.3 Results and Discussion

There is strong epidemiological data that alcohol can act as a tumor promoter. Chronic alcohol consumption increases the risk of HCC a further 2-fold when combined with factors such as HCV/HBV infection or diabetes [2]. We have developed a mouse model which replicates this phenomenon in which increased adenoma multiplicity occurs in mice where tumors are initiated with DEN on PND 13 and EtOH is administered chronically for 16 weeks in Lieber DeCarli liquid diets beginning in adulthood [4, 7]. We have previously shown that at a final concentration of 28% total calories, the blood alcohol concentrations attained in this model average 75 mg/dL which is comparable to the human 80 mg/dL limit for DWI [7]. Tumor promotion was accompanied by appearance of steatohepatitis, fibrosis and stimulation of Wnt-β-cantenin-dependent hepatocyte proliferation coincident with loss of hepatic retinoids [4, 7]. In the current study, we examined the possible protective effects of feeding soy protein isolate and of a major soy-associated phytochemical genistein in the DEN-EtOH mouse tumor promotion model based on literature showing ant-tumorigenic effects and inhibition of Wnt-β-catenin signaling in other cancer models. As expected, chronic EtOH feeding of adult mice as part of Lieber DeCarli liquid diets resulted in appearance of adenomas in 81% of DEN-treated mice with a multiplicity of 2 tumors/mouse (Fig. 9.1). When the protein source in the EtOH-Lieber DeCarli diet was switched from casein to SOY, adenoma incidence was reduced to 26% and multiplicity was reduced by 75% ($P < 0.05$) (Fig. 9.1). Surprisingly, supplementation of the EtOH-Lieber DeCarli diet with 250 mg/kg diet genistein, a level comparable to that found in SOY actually increased adenoma incidence to 92% and multiplicity to 5 tumors/mouse ($P < 0.05$). These data suggest that SOY contains factors that inhibit EtOH-induced tumor promotion but that the bioactive component may be either a phytochemical other than genistein or a protein/peptide. SOY contains over 100 phytochemicals and peptides and the major soy storage protein β-conglycenin may also give rise to bioactive peptides after digestion [23, 24]. Identification of this cancer protective component of SOY remains the subject of future studies.

We conducted further analyses to examine the molecular mechanisms underlying the promotional protection afforded by feeding SOY with EtOH. In saline-treated mice, the EtOH/SOY group had significantly reduced necroinflammatory injury and Kupffer cell activation with lower levels of serum ALT, hepatic mRNA expression of CD14, TNFα, IL-6, CXCL-2 and nuclear expression of the NFκB p65

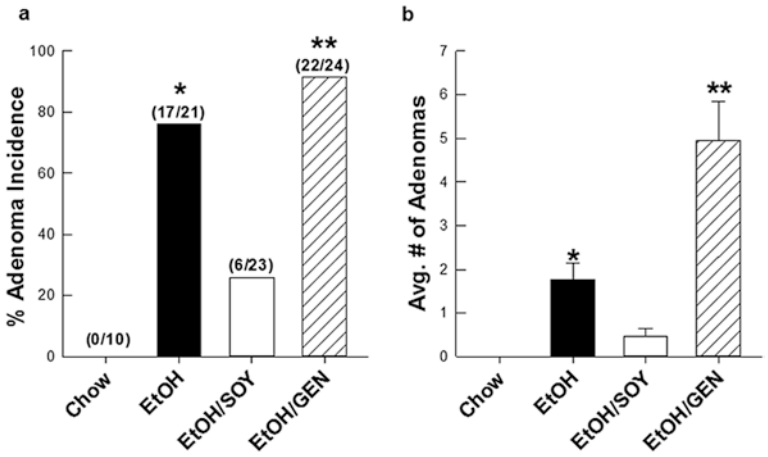

Fig. 9.1 Adenoma incidence (**a**) and tumor multiplicity (**b**) in DEN-treated male mice receiving a standard LieberDeCarli EtOH liquid diet using casein (EtOH) or soy protein isolate (EtOH/SOY) as the sole protein source as previously published [29]; and with a third group (EtOH/GEN) receiving the EtOH liquid diet supplemented with (250 mg/kg diet) genistein for 16 wks. Data expressed as mean ± SEM. Adenoma incidence was determined using Fisher's Exact Test. Multiplicity was determined One-Way ANOVA followed by Mann-Whitney U rank-sum test for post hoc comparisons, *p < 0.05 EtOH vs. EtOH/SOY, **p < 0.05 EtOH vs. EtOH/GEN

Table 9.2 Biochemical analysis of liver injury and inflammatory response in saline-treated male mice receiving EtOH or EtOH/SOY diets for 16 wks [29].

	ALT	TNFα mRNA expression	IL6 mRNA expression	CD 14 mRNA expression	CXCL2 mRNA expression	NFκB nuclear expression
Chow	8.63 ± 0.89^{a}	0.15 ± 0.03^{a}	0.05 ± 0.12^{a}	1.31 ± 0.27^{a}	0.09 ± 0.01^{a}	–
EtOH	37.65 ± 3.62^{b}	2.36 ± 0.51^{b}	2.26 ± 0.34^{b}	43.37 ± 0.37^{b}	0.91 ± 0.15^{c}	1.07 ± 0.16
EtOH/SOY	8.67 ± 1.58^{a}	1.23 ± 0.21^{a}	0.72 ± 0.13^{a}	2.71 ± 0.29^{a}	$0.46 \pm 0.11^{a,b}$	$0.55 \pm 0.08^{*}$

Data is expressed as mean ± St.Err; Groups: chow (n = 5), EtOH (n = 10), EtOH/SOY (n = 10). Liver injury was assessed by measuring serum alanine transferase (ALT), in S.F. units/ml [30]. Gene expression was determined by real-time RT-PCR as previously described [29], Significance, a<b<c, (p<0.05).

subunit when compared to the EtOH group (P < 0.05) (Table 9.2). EtOH feeding increased hepatic concentrations of ceramide and hepatic mRNA expression of enzymes involved in synthesis of ceramide, sphingosine and sphingosine-1-phosphate (S1P); (ceramide synthase 1 – CERS1; acid ceramidase – ASAH1; serine palmitoyl transferase and sphingosine kinase-1 –SPKH1); compared to chow controls (P < 0.05) and increased SPKH1 protein expression (Table 9.3, Fig. 9.2). In addition mRNA encoding S1P receptors S1PR2 and S1PR3 were increased in the EtOH group relative to chow controls (Table 9.3). In contrast, feeding SOY with EtOH

Table 9.3 Changes in hepatic sphingosine signaling mediators in response to EtOH and EtOH/SOY diets in saline-treated male mice [29].

	SPKH1 membrane expression	mRNA expression (fold change)				
		SPKH1	ASAH1	S1PR1	S1PR2	S1PR3
Chow	–	1.00 ± 0.05^{a}	1.00 ± 0.04^{a}	1.00 ± 0.16^{a}	1.00 ± 0.10^{a}	1.00 ± 0.14^{a}
EtOH	1.60 ± 0.15	23.45 ± 5.67^{b}	$1.70 \pm 0.29^{a,b}$	0.96 ± 0.22^{a}	3.59 ± 0.93^{b}	2.64 ± 0.54^{b}
EtOH/SOY	$0.70 \pm 0.08^{*}$	9.12 ± 2.20^{a}	0.79 ± 0.17^{a}	0.54 ± 0.09^{a}	1.31 ± 0.28^{a}	1.36 ± 0.30^{a}

Data is expressed as mean ± St.Err; Groups: chow (n=5), EtOH (n=10), EtOH/SOY (n=10). Gene expression and protein determination were determined as previously described (Mercer et al. 2016), Significance, a<b<c, ($p<0.05$), Student's T-Test, *$p<0.05$.

blocked the effects of EtOH on CERS1 mRNA and reduced hepatic ceramide concentrations compared to the EtOH group ($P < 0.05$) (Fig. 9.2). In addition, the EtOH/SOY group had lower SPKH1 mRNA and protein and reduced expression of S1PR2 and S1PR3 mRNA (Table 9.3). As previously reported, coincident with its promotional effects, EtOH increased hepatocyte proliferation in a β-catenin-dependent manner. EtOH increased expression of cyclin D1 mRNA and mRNA expression of other downstream β-catenin targets MMP7 and glutamine synthase ($P < 0.05$) (Fig. 9.3). In saline-treated mice, feeding SOY with EtOH reversed these effects ($P < 0.05$). Relative the EtOH group, the EtOH/SOY group had lower nuclear β-catenin protein ($P < 0.05$) (Fig. 9.3) and PCNA staining of proliferating hepatocytes was reduced from 2.5 ± 0.4 to 0.9 ± 0.1% ($P < 0.0.5$). These data suggest that reduction in EtOH-dependent tumor promotional stimuli after SPI feeding are linked to lower necroinflammatory injury and normalization of ceramide/sphingosine signaling. Proliferative and regenerative repair responses are generally observed in the liver after injury [25]. It has been suggested that hepatocyte proliferation is a Wnt-regulated process linked to reduced retinoid signaling [5]. Sphingosine 1-phosphate signaling has been shown to negatively cross talk with retinoids and has been shown to activate hepatic stellate cells [26, 27]. Moreover, Wnt signals from other hepatic cell types including Kuffper cells have been shown to regulate hepatocyte proliferation under conditions of partial hepatectomy [28]. It remains to be seen if similar signals from activated Kupffer or stellate cells regulate hepatocyte and hepatic tumor cell proliferation after EtOH consumption and if the molecular mechanisms whereby SOY prevents these mechanisms are related to its effects on ceramide/sphingosine signaling pathways.

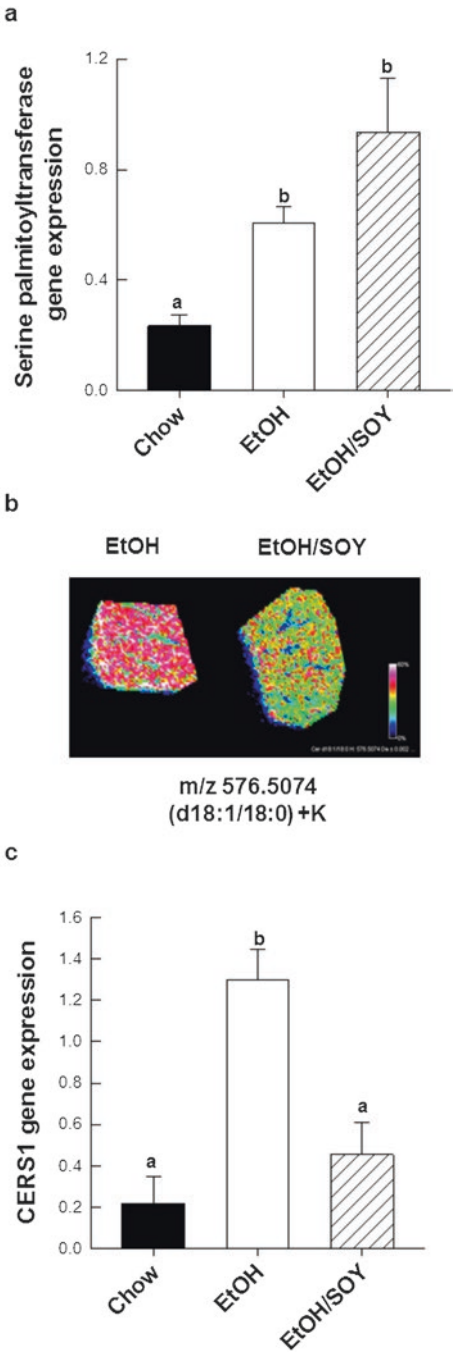

Fig. 9.2 Alcohol feeding increased de novo ceramide synthesis as demonstrated by increased mRNA expression of (**a**) serine palmitoyltransferase; EtOH-specific increases in C18 ceramide (d18:1/18:0) as observed by (**b**) high resolution MALDI-FTICR imaging mass spectrometry, and (**c**) increased ceramide synthase 1 mRNA expression were prevented in EtOH/SOY-treated mice [29]. Significance $a < b < c$, ($p < 0.05$)

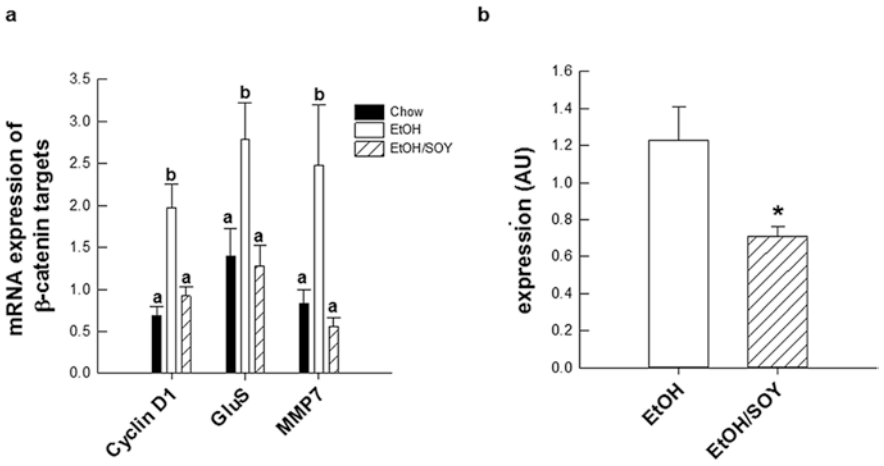

Fig. 9.3 Changes in mRNA expression of (**a**) β-catenin targets, cyclin D1, GluS, and MMP7, and (**b**) nuclear expression of β-catenin in saline-treated mice receiving the EtOH or EtOH/SOY diet for 16 wks. Significance a < b < c, ($p < 0.05$), Student T-test, *$p < 0.05$ [29]

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Chapter 10
ALDH1L1 and ALDH1L2 Folate Regulatory Enzymes in Cancer

Sergey A. Krupenko and Natalia I. Krupenko

Abstract Epidemiological studies implicate excess ethanol ingestion as a risk factor for several cancers and support the concept of a synergistic effect of chronic alcohol consumption and folate deficiency on carcinogenesis. Alcohol consumption affects folate-related genes and enzymes including two major folate-metabolizing enzymes, ALDH1L1 and ALDH1L2. ALDH1L1 (cytosolic 10-formyltetrahydrofolate dehydrogenase) is a regulatory enzyme in folate metabolism that controls the overall flux of one-carbon groups in folate-dependent biosynthetic pathways. It is strongly and ubiquitously down-regulated in malignant tumors via promoter methylation, and recent studies underscored this enzyme as a candidate tumor suppressor and potential marker of aggressive cancers. A related enzyme, ALDH1L2, is the mitochondrial homolog of ALDH1L1 encoded by a separate gene. In contrast to its cytosolic counterpart, ALDH1L2 is expressed in malignant tumors and cancer cell lines and was implicated in metastasis regulation. This review discusses the link between folate and cancer, modifying effects of alcohol consumption on folate-associated carcinogenesis, and putative roles of ALDH1L1 and ALDH1L2 in this process.

Keywords Folate · Cancer · Alcohol · ALDH1L1 · ALDH1L2 · Methylation · SNPs · Tumor suppressor

10.1 Introduction

Epidemiological studies implicate excess ethanol ingestion as a risk factor for several cancers and support the concept of a synergistic effect of chronic alcohol consumption and folate deficiency on carcinogenesis [1]. Alcohol consumption

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itself impairs folate metabolism through the enhanced coenzyme degradation or the inhibition of absorption, as well as through the influence on folate-related genes and enzymes [1, 2]. Among these targets, two major folate-metabolizing enzymes, ALDH1L1 and ALDH1L2, were considered. This review discusses the link between folate and cancer, modifying effects of alcohol consumption on folate-associated carcinogenesis, and putative role of ALDH1L1 and ALDH1L2 in this process.

10.2 Folate: Overview

Folate coenzymes are vital for cellular homeostasis due to their key role in transferring one-carbon groups in reactions of *de novo* nucleotide biosynthesis, metabolism of serine, glycine and histidine, and the regeneration of methionine from homocysteine [3]. The methionine biosynthesis is linked to the production of S-adenosylmethionine, a universal methyl donor in more than a hundred methylation reactions in the cell [4]. Folates also participate in the clearance of formate as CO_2 [5] and the formylation of methionyl-tRNA [3]; the latter process is essential for translation initiation in eukaryotic mitochondria [6]. Humans are unable to synthesize this coenzyme and must obtain it from the diet. Insufficient folate intake has dramatic consequences for the cell, including: deregulation of methylation processes [7]; altered protein expression [8]; and decreased DNA repair capability and accumulation of DNA damage leading to increased chromosomal aberrations and fragility [9, 10]. These mechanisms underlie reduced growth rate and impaired cell division caused by folate deficiency. Low folate status has been linked to increased risk for several types of cancer, neural tube defects, and cardiovascular diseases [7] though the association between folate and carcinogenesis, as well as cardiovascular diseases, is inconclusive at present. For the reason of the prevention of neural tube defects, in 1996 the FDA approved a mandatory fortification of several types of grain foods in the US with a synthetic form of the vitamin, folic acid. Though the overall importance of folate for human health was known for long time, the underlying molecular mechanisms are not fully understood and continue to emerge. This is exemplified by recent studies, which have underscored the significance of folate metabolism for ES cells [11], the contribution of folate-dependent carbon oxidation into the cellular energy balance [12, 13], and the role of folate enzymes in cancer progression and metastasis [14–19]. To further complicate the picture, a concept that parental folate intake or folate status can modify disease risk in offspring later in life has been recently proposed [20].

10.3 Interactions Between Alcohol Consumption and Dietary Folate: Implication for Cancer

The link between folate and cancer has been investigated for decades but this issue is complicated by the phenomenon that while in general folate intake protects against tumorigenesis, it also can promote the proliferation of existing neoplastic lesions [21]. This adverse effect is primarily associated with the increased demand of rapidly proliferating cancer cells for folate coenzymes to support enhanced nucleotide biosynthesis towards unlimited DNA replication. Thus, there is a dilemma that folate intake above basal requirements may increase the incidence of malignancies and cancer-related death, which has been increasingly recognized by the experts in folate field [22]. The tumorigenic response to dietary folate depends on numerous factors, including cancer subtypes, the timing or duration of vitamin administration, its dose and ingested form (synthetic folic acid *vs*. natural folate) [4, 21]. End-point effects of the vitamin could be further modified by other factors such as age, the status of vitamins B_6/B_{12}, and individual genotypic features including polymorphisms in folate enzymes [4, 20]. One of the factors known to affect folate metabolism is chronic alcohol consumption [1, 2].

Alcoholism is typically associated with folate deficiency due to reduced dietary folate intake [2]. Heavy alcohol consumption also decreases folate absorption, enhances urinary folate excretion and inhibits enzymes pivotal for one-carbon metabolism [1, 2]. While folate metabolism is involved in numerous key biochemical pathways (Fig. 10.1), the aberrant DNA methylation, due to the deficiency of methyl donors, was widely considered as a common downstream target of the folate-mediated effect of ethanol [23]. The negative effects of low intakes of nutrients, which provide dietary methyl groups, with high intakes of alcohol are additive in general [24]. In support of such association, it has been reported that the low methionine, low-folate diets and alcohol consumption increase the risk for colorectal cancer in men [25]. Therefore, to counteract the negative effects of alcohol consumption, the increased intake of nutrients providing dietary methyl groups is recommended [24].

In agreement with this notion, a protective effect of folate on alcohol-impaired processes has been demonstrated in experiments with cultured mouse embryos, where addition of the vitamin, in the form of folic acid, blocked ethanol-induced teratogenesis [26]. The microarray profiling further indicated that the effect of prenatal ethanol exposure on teratogenesis in mice, and associated mental retardation, were induced through alterations in the expression pattern of several micro RNAs in fetal brain. In line with this mechanism, increased folic acid prevented micro RNAs changes in response to ethanol. Though it is not clear whether a similar mechanism mediating the interaction between dietary folate and alcohol consumption could be activated in carcinogenesis, SNPs (single nucleotide polymorphisms) in the micro RNA bindings sites of thymidylate synthase were associated with gastric cancer risk and patient survival [27]. Perhaps the interaction between folate status and alcohol consumption in carcinogenesis involves multiple mechanisms and is likely cancer-type specific. Four main alcohol-associated cancers are liver, colon, breast

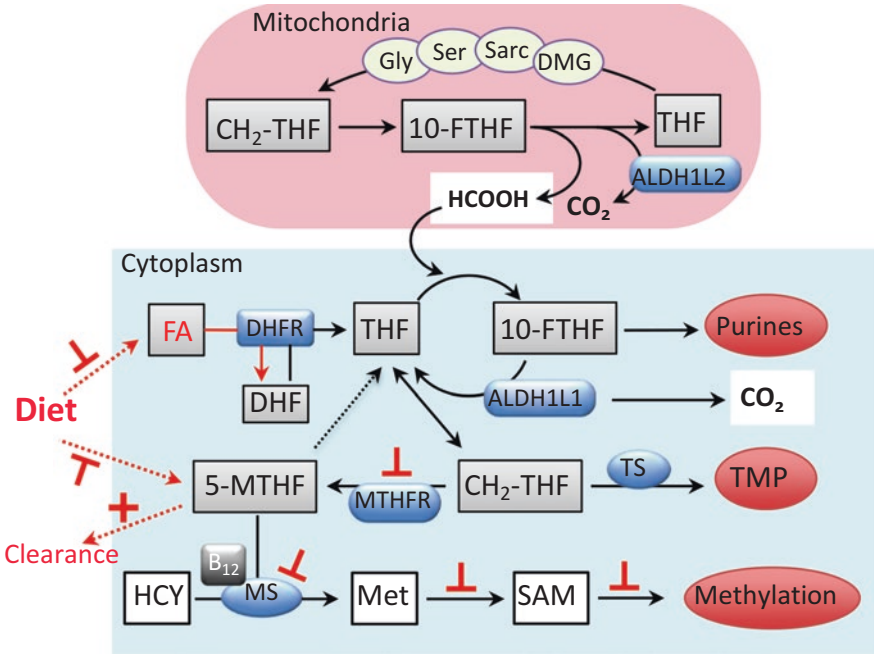

Fig. 10.1 ***Folate metabolism.*** Folate is taken up by the cell as folic acid (FA, supplements or fortified foods) or 5-methyl-THF (5-MTHF, natural diet). In the cell, FA is sequentially converted to dihydrofolate (DHF) and the active form of the coenzyme, tetrahydrofolate (THF) in reactions catalyzed by DHFR (dihydrofolate reductase). Acceptance of a one-carbon group (comes from serine, glycine, histidine or formate) converts THF to coenzymes directly participating in biosynthetic reactions (10-FTHF, 10-formyl-THF; CH_2-THF, 5,10-methylene-THF). HCY, homocysteine; SAM, S-adenosylmethionine; MS, methionine synthase; TS, thymidylate synthase; MTHFR, methylenetetrahydrofolate reductase. Reaction catalyzed by MS converts 5-MTHF to THF (*dotted arrow*). Overall, folate coenzymes provide one-carbon groups for three biosynthetic pathways: (i) methionine production; (ii) *de novo* purine generation; (iii) TMP synthesis. Mitochondrial folate metabolism provides one-carbon groups, derived from the degradation of serine, glycine, sarcosine (Sarc) or dimethylglycine (DMG), to the cytosolic folate pathways in the form of formate. Processes inhibited by ethanol are indicated by (⊥). Degradation of 5-methyl-THF is accelerated by ethanol (indicated by "+")

and upper aerodigestive tract [23]. In agreement with such etiology, recent prospective cohort study indicated that the folate pathway is likely to be involved in alcohol-related colorectal cancer development [28]. Higher folate intake can also ameliorate the effect of alcohol consumption on the development of HCC (hepatocellular carcinoma) [29] and the risk of breast cancer [30]. A prospective study of alcohol consumption and the risk of colorectal cancer before and after folic acid fortification in the US showed that fortification may attenuate this risk [31]. Another case-control study indicated that folate-related enzyme polymorphisms modify the association between drinking habit and pancreatic cancer risk [32]. Studies of other cancer types did not provide a clear association between folate status, alcohol consumption and cancer risk [33, 34].

The effect of ethanol on folate metabolism could be direct, through the enhanced coenzyme degradation or the inhibition of absorption, as well as indirect, through the influence on folate-related genes and enzymes. The intracellular folate pool consists of several major forms of the coenzyme, which differ in the oxidation level of the bound one-carbon group and are interconvertible through multiple reactions catalyzed by more than a dozen enzymes (Fig. 10.1). Enzymes of folate metabolism bring one-carbon groups to the folate pool, oxidize/reduce the folate-bound groups, or utilize these groups in biosynthetic reactions. The role of folate enzymes in cancer is well established and some of them, including DHFR and thymidylate synthase, are canonical chemotherapeutic targets [35]. An additional association between folate enzymes and cancer is provided by epidemiologic studies, which linked SNPs in the *MTHFR* gene with the risk of several cancer types [23]. The combination of folate intake and SNPs in genes associated with methionine biosynthesis may contribute to breast [36] and gastric [37] cancer risk, indicating that folate intake-associated cancer risk can be further modified by gene-nutrient interactions. Towards this line, a cross-sectional analysis of 19 human studies indicated a role for folate enzymes and their SNPs in response to alcohol consumption [38]. The direct inhibitory effect of ethanol on the activities of MTHFR and MTR in an animal model was demonstrated as well [39]. As a likely cause of decreased liver SAM and reduced methylation capacity, this mechanism can contribute to carcinogenesis [23]. Of note, ethanol also decreases thymidylate synthase mRNA levels in regenerating liver after partial hepatectomy [40], the effect which could be translated into the impaired DNA synthesis and repair.

10.4 ALDH1L1 Role in Cancer

One of the most abundant folate enzymes is cytosolic 10-formyltetrahydrofolate dehydrogenase (FDH, ALDH1L1) [41]. Levels of this enzyme can reach about 1.2% of the total protein in rat liver cytosol [42, 43], suggesting an important role (proposed functions for the enzyme are summarized in Fig. 10.2). ALDH1L1 converts 10-formyl-THF to THF (tetrahydrofolate) and carbon dioxide in a $NADP^+$-dependent reaction (Fig. 10.2). This reaction clears one-carbon groups (in the form of CO_2) from the cell thus limiting their flux toward folate-dependent biosynthetic reactions (Fig. 10.1) [44, 45]. It is also important for replenishing the pool of THF [46], which is the only folate coenzyme capable of accepting one-carbon groups and thus is central to folate metabolism [47]. In agreement with such function of ALDH1L1, genome-wide association studies revealed that SNPs in this gene are associated with serine to glycine ratio in serum [48] (THF is required for the reaction of the conversion of serine to glycine, Fig. 10.1). Furthermore, ALDH1L1 might regulate *de novo* purine biosynthesis [44, 49], formate degradation [5] and methylation status of the cell [45]. Another function originally proposed for this enzyme is to serve as the folate depot, though this hypothesis is primarily based on the phenomenon that the protein was purified in complex with THF [42].

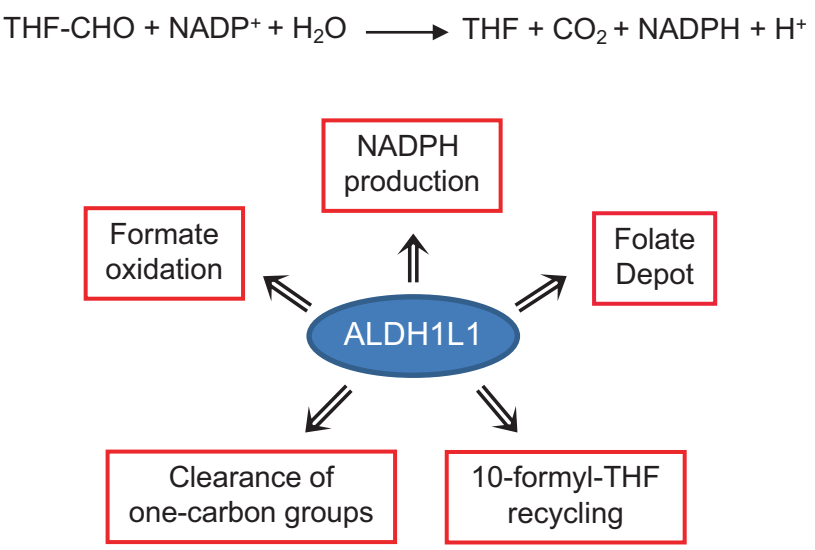

Fig. 10.2 Reaction catalyzed by ALDH1L1 and ALDH1L2 and proposed biological roles for the enzymes. *THF* tetrahydrofolate; *THF-CHO* 10-formyl-THF

ALDH1L1 is not ubiquitously expressed in human tissues: highest levels of its mRNA were detected in liver, kidney and pancreas while the levels in several tissues including placenta, spleen, thymus, small intestine, leukocytes, testis, and ovary were undetectable [44, 50]. Interestingly, ALDH1L1 is also differentially expressed in central nervous system during development: most quiescent cells in developing mouse brain are ALDH1L1 positive while proliferating cells do not express this protein [51]. Curiously, levels of this protein also significantly fluctuate (up to about seven-fold change) in the liver of golden-mantled ground squirrel depending on seasonal stages [52]. In further support of highly regulated expression of this protein, its levels were decreased in rat liver by clofibrate, a peroxisome proliferator [53] and increased in zebrafish embryos exposed to ethanol [54].

Perhaps most striking example of the ALDH1L1 regulation is its silencing in malignant tumors [44], which is achieved through methylation of the CpG island within the *ALDH1L1* promoter [55]. It contains 96 CpG pairs and covers the region between −525 and + 918 bp of the *ALDH1L1* gene including the promoter, the entire exon 1, and a part of intron 1 immediately downstream of the exon. Bisulfite sequencing analysis revealed extensive methylation of the island (76%–95% of CpGs) in cancer cell lines. Analysis of the samples from patients with lung adenocarcinomas demonstrated methylation of the *ALDH1L1* CpG island in tumor samples and a total lack of methylation in respective normal tissues. The same phenomenon was observed in liver tissues: the CpG island was methylation free in DNA extracted from normal hepatocytes but was extensively methylated in a hepatocellular carcinoma. Levels of *ALDH1L1* mRNA and protein correlated with the methylation status of the island, with tumor samples demonstrating down-

regulation of expression or even complete silencing of the gene. The down-regulation of *ALDH1L1* mRNA in NSCLC (non-small cell lung cancer) [56], cervical cancer [57] and renal cell carcinoma [58] associated with the gene methylation was also demonstrated by microarray assays. The regulation of *ALDH1L1* through the promoter methylation could also be a common cellular response to the environmental conditions. Thus, it has been reported that the prolonged exposure to isoflavone through dietary supplementation significantly reduces *Aldh1l1* promoter methylation in rat mammary tissue [59]. In addition, the methylation of *ALDH1L1* could be responsible for the individual variation in the protein expression. For example, higher CpG methylation in the body of the *ALDH1L1* gene was significantly correlated with its lower transcript expression in normal breast tissue in women [60].

In agreement with the phenomenon that ALDH1L1 is down-regulated in proliferating tumors, re-expression of the protein in cancer cells produces drastic antiproliferative effects including cell cycle arrest and apoptosis [44, 49, 61–63]. These findings indicated that ALDH1L1 is a key regulator of proliferation and an implication has been made in the literature that this protein is a candidate tumor suppressor [44, 55, 57, 58, 64]. Furthermore, under-expression of this gene could be a marker of a more aggressive tumor phenotype. Thus, decreased expression of ALDH1L1 was associated with aggressive subtypes of sporadic pilocytic astrocytoma [64], poor prognosis in hepatocellular carcinoma [65], and low overall survival in neuroblastoma [66], while high expression of *ALDH1L1* mRNA correlates with better overall survival in breast cancer patients [67]. It should be mentioned that the association between decreased ALDH1L1 expression and malignant tumor progression could be cancer type-specific [68]. For example, though decreased expression of ALDH1L1 was demonstrated in NSCLC [55, 56], cervical cancer [57], renal cell carcinoma [58] and peripheral cholangiocarcinoma [69], the extent of its expression in other cancers is not clear. In line with the idea that ALDH1L1 prognostic role could be cancer type-specific, SNPs in the *ALDH1L1* gene were significantly associated with altered risk of breast cancer [70] and increased risk of hepatocellular carcinoma [71] and non-Hodgkin lymphoma [72–74] but no SNPs were associated with the risk of prostate cancer [75].

10.5 Role of Mitochondrial ALDH1L2 Enzyme in Cancer

Folate pathways are compartmentalized within the cell, mainly between cytoplasm and mitochondria [3, 76], though the compartmentalization was recently extended to the nucleus, where folate-dependent TMP biosynthesis takes place [77]. It has been suggested that the mitochondrial pathways mainly serve to provide one-carbon groups, in the form of formate, for incorporation into the cytosolic folate pool where they are utilized for biosynthetic purposes [3]. While some folate-dependent reactions are unique to cytoplasm or mitochondria, several of them take place in both compartments and are catalyzed by homologous enzymes, which are products of distinct genes [3, 78]. The oxidation of 10-formyl-THF to THF and CO_2 is one of

such reactions. Mitochondrial 10-fTHF dehydrogenase is encoded by the *ALDH1L2* gene, which originated via the duplication of the *ALDH1L1* gene and acquired a mitochondrial leader sequence [50]. Accordingly, two proteins share about 72% identity of the amino acid sequence and are close structurally and enzymatically [50, 79]. Their biological roles, however, could be quite different. While cytosolic ALDH1L1 is involved in the regulation of cellular proliferation, through the control of folate pools, ALDH1L2 is the key enzyme to provide reduced NADPH in mitochondria [12]. NADPH produced in this mitochondrial reaction is required for the reduction of oxidized glutathione, and the loss of ALDH1L2 shifts the ratio of GSH/GSSG. This in turn decreases the capacity of mitochondria to eliminate reactive oxygen species leading to oxidative stress [19].

The *ALDH1L2* gene was discovered relatively recently and its studies are limited so far. Of note, it can be up-regulated by certain drugs, though mechanism of this response were not studied. For example, *ALDH1L2* mRNA levels are strongly increased (up to 6.8-fold) in immortalized human B cells treated with ER stress inducers thapsigargin or tunicamycin [80]. *ALDH1L2* mRNA was also significantly up-regulated in human adrenocortical NCI-H295R cells treated with mitotane, an adrenolytic drug extensively used in combination with other cytotoxic drugs and as an adjuvant monotherapy in the treatment of adrenocortical carcinoma [81]. This effect, however, is hard to correlate with the pharmacological action of the drug. *ALDH1L2* mRNA was also up-regulated more than three-fold in mouse neonatal ovaries exposed to 3-methylcholanthrene, a potent ovotoxicant [82]. The question of whether the regulatory effects of these drugs on the *ALDH1L2* gene are associated with the cellular response to oxidative stress awaits further investigation. Curiously, the *ALDH1L2* gene expression was lost in CCL-131 Neuro-2a malignant neuroblastoma cells at acidic pH [83]. Likewise, levels of the ALDH1L2 protein were dramatically decreased in nonalcoholic steatohepatitis in rats fed fat-rich diet [84]. Another study has reported a different effect for ALDH1L2: the protein was elevated about two-fold in fibroblasts of the patient with short-chain acyl-CoA dehydrogenase deficiency [85]. Thus, it appears that levels of ALDH1L2 inversely correlate with fatty acid oxidation. It has been also suggested that both acidic pH and fatty acid oxidation deficiency induce metabolic reprogramming, driving the switch to OXPHOS and less glucose utilization in the former case and to biosynthetic processes in the latter case. In this regard, ALDH1L2 could be differentially regulated depending on the cellular demand for the energy production. Alternatively, the regulation could be driven by ROS levels as well as the ratio of reduced/oxidized glutathione but precise mechanisms controlling ALDH1L2 expression remain elusive.

Of note, ALDH1L2 has a different tissue-expression pattern than ALDH1L1 and in contrast to the cytosolic enzyme is highly expressed in cancer cell lines [50]. It has been recently reported that ALDH1L2 is up-regulated in human colorectal tumor tissues compared to normal tissues [86]. Furthermore, rates of recurrence-free survival and overall survival in patients with high expression of ALDH1L2 tend to be lower than in patients with low expression of the enzyme, the situation opposite to cytosolic ALDH1L1. Considering that ALDH1L2 is a mitochondrial protein, it should be pointed out that numerous recent studies specifically underscored the

role of mitochondrial folate pathways in cancer with the emphasis on folate-dependent metabolism of serine and glycine [14–16, 87–90]. In this regard, ALDH1L2 might be an important component providing THF for the serine to glycine conversion in mitochondria and for glycine degradation (Fig. 10.1).

Intriguingly, *ALDH1L2* was implicated as a metastasis-regulatory gene [18]. Thus, in a mouse melanoma metastasis model, a striking increase in the expression of this protein in liver, pancreas and lung metastases compared to subcutaneous tumors has been shown [18]. Of note, other folate enzymes tested in this study did not demonstrate such trend. In further support of the metastasis-promoting role of ALDH1L2, the silencing of the gene in melanoma by shRNA significantly reduced the frequency of circulating melanoma cells in blood and overall metastatic burden [18]. Interestingly, the reduced invasion of MDA-MB-435 cells after the treatment with anti-inflammatory agent indomethacin was associated with a significant elevation of *ALDH1L2* mRNA [91]. While the mechanism by which indomethacin leads to *ALDH1L2* gene up-regulation is not clear, it could be a compensatory cellular response to the increased ROS production caused by the drug. The cytosolic isoform, ALDH1L1, could be also associated with the metastatic potential of cancer cells [92]. In contrast to ALDH1L2, however, the cytosolic isoform inhibits cellular migration and invasion, the phenomenon rather associated with the decreased metastasis [93].

10.6 Effect of Ethanol on ALDH1L1/L2 Genes and Proteins

One of the folate-related effects of alcohol consumption could be the interaction of ethanol or its metabolites with folate enzymes [39]. Since the decrease of the ALDH1L1 expression could be associated with tumor promotion, metabolites inhibiting the activity of the enzyme or causing down-regulation of its expression would have pro-tumorigenic effect. Though studies of the effect of ethanol on ALDH1L1 are scarce, a role for the enzyme in mediation of the effect of alcohol intake on oral carcinogenesis has been proposed [94]. In another study, levels of ALDH1L1 were changed after liver transplant in recipients with alcoholic cirrhosis [95], implying the effect of chronic alcohol consumption on the enzyme. Interestingly, a recent study reported that alcohol consumption is associated with differentially methylated CpGs in the *ALDH1L1* gene in breast tissue of healthy women [60]. Furthermore, women carrying an allelic variant of the gene were more likely to have hypermethylated *ALDH1L1*, the phenomenon correlated with lower gene expression. These findings point toward a potential mechanism by which alcohol implements its folate-mediated tumorigenic effect in mammary tissue.

Chronic ethanol ingestion was reported to decrease hepatic ALDH1L1 dehydrogenase activity in rats [96]. The ethanol treatment also affected ALDH1L1 activities in brain and hepatic tissues of chicken embryos [97, 98]. It has been further demonstrated that the ALDH1L1 enzymatic activity is inhibited by acetaldehyde *in vitro* [99], which could be a mechanism of the ethanol effect on the enzyme and one of the mechanisms by which alcohol consumption changes folate status. It should be

noted that ALDH1L1 is capable, at least *in vitro*, to metabolize acetaldehyde to acetic acid [100], which would argue against such inhibitory effect. This reaction still could interfere with the folate-related catalysis of the enzyme thus affecting folate metabolism. ALDH1L1 has been also reported as a target for acetaminophen, which covalently modifies a key cysteine of the enzyme; this effect could contribute to the drug toxicity [101]. Since excessive alcohol consumption is a risk factor for acetaminophen-induced hepatotoxicity [102], ALDH1L1 could be a dual target towards liver damage. Whether this effect would contribute to carcinogenesis is not clear at present. Interestingly, ALDH1L1 can also counteract the effect of ethanol on folate metabolism by protecting THF from degradation [103]. The metabolism of acetaldehyde by xanthine oxidase generates superoxide radicals, which can cleave folates [104]. Of note, 5-methyl-THF, the most common form of natural folate, is highly susceptible to the degradation by superoxide [104]. In agreement with the mechanism of folate protection by ALDH1L1, up-regulation of the *ALDH1L1* gene prevented folate degradation and alleviated the oxidative stress induced by ethanol exposure in zebrafish embryos [54].

The role of ALDH1L2 in alcohol response and ethanol metabolism is even less clear due to the lack of corresponding studies. By analogy with the *ALDH1L1* gene, it can be hypothesized that ALDH1L2 is relevant to the interaction between ethanol and folate metabolism. Indeed, the implication that this gene is a part of alcohol dependence mechanism has been made in the literature [105]. Thus, in the study of genome-wide DNA methylation in discordant sib pairs with alcohol dependence, the deregulation of *ALDH1L2* gene through the promoter hypomethylation was associated with alcohol dependence [105].

10.7 Conclusion

ALDH1L1 and ALDH1L2 are key enzymes in the regulation of folate metabolism as well as downstream processes associated with folate-dependent biochemical reactions. While both enzymes catalyze the same reaction, their compartmentalization leads to the differential effect on overall cellular metabolism, regulating either reduced folate pools and purine biosynthesis (cytosolic ALDH1L1) or NADPH production and oxidative stress (mitochondrial ALDH1L2). Both enzymes were implicated in the proliferation of malignant tumors, though with opposite roles, tumor suppression in the case of the cytosolic enzyme and metastasis promotion in the case of the mitochondrial isoform. These enzymes were also implicated in the cellular response to alcohol consumption. Taking into account that both enzymes have essentially identical structural organization and enzymatic mechanism, it is likely that the direct effect of ethanol or its metabolites on ALDH1L1 and ALDH1L2 would be similar in both cases. However, considering differential regulation of the two isoforms, the overall effect of alcohol consumption on two enzymes would be more complex and not so direct. Clearly, more studies are needed to address the role of ALDH1L1 and ALDH1L2 in biology of malignant tumors and in potential mediation of the alcohol effect.

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Chapter 11
Developmental Morphogens & Recovery from Alcoholic Liver Disease

Anna Mae Diehl

Abstract Alcohol-induced steatohepatitis (ASH) increases the risk for both clinically-severe acute alcoholic hepatitis and eventual cirrhosis. The mechanisms that control ASH pathogenesis and progression are unclear but processes that regulate liver cell plasticity seem to be critically involved. In injured adult livers, morphogenic signaling pathways that modulate cell fate decisions during fetal development and in adult liver progenitors become reactivated. Overly-exuberant activation of such morphogenic signaling causes dysregulated liver repair and increases short- and long-term mortality by promoting acute liver failure, as well as progressive fibrosis. Hence, these pathways may be novel therapeutic targets to optimize liver cell reprogramming and prevent defective regenerative responses that cause acute liver failure and cirrhosis.

Keywords Steatohepatitis · ASH pathogenesis · Cirrhosis · Liver failure

Alcoholic liver disease encompasses a spectrum of liver pathology with very different prognoses [1]. Steatosis (i.e., lipid accumulation in hepatocytes) is a common consequence of chronic alcohol consumption and it generally has a benign liver prognosis. However, some individuals with steatosis develop hepatic inflammation, hepatocyte injury and death, a condition termed steatohepatitis. Steatohepatitis is a dynamic process. It can regress to steatosis, smolder indolently for decades, or incite progressive fibrosis that ultimately results in cirrhosis. Steatohepatitis per se is typically not associated with overt manifestations of liver disease and thus, often is diagnosed only after liver damage has advanced to cirrhosis with portal hypertension. However, sometimes individuals with steatohepatitis exhibit florid features of acute liver failure with extreme liver-related morbidity and 30 day mortality rates as high as 30% [2]. This clinical syndrome has been dubbed acute alcoholic hepatitis.

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The factors that determine the course of alcohol-related liver pathology are not well-understood but clarifying this issue is critical in order to develop effective interventions to prevent acute alcoholic hepatitis and cirrhosis, the two main causes of alcohol-related liver morbidity and mortality.

Differences in risk for liver-related death can be used to segregate alcoholic liver disease into two major subgroups: isolated steatosis (which has a generally good prognosis) and steatohepatitis (which increases risk for acute and chronic liver failure). Liver cell injury and death distinguish steatohepatitis from steatosis, suggesting that dying hepatocytes may determine liver outcomes in alcoholic liver disease. This concept is plausible given that organ failure generally results when cell death exceeds cell replacement. To avoid liver failure, dying hepatocytes release signals that trigger their replacement and promote eventual recovery of functional liver parenchyma [3]. These wound healing responses are multi-faceted and transiently enrich the liver with regenerative cell types that are not abundant in healthy livers. For example, inflammatory cells are recruited to clear death-related debris, endothelial cells are activated for vasculogenesis to optimize tissue blood flow, myofibroblasts accumulate to remodel the matrix to support repair, and liver progenitors emerge and differentiate to replace dead hepatocytes. An orderly progression of wound healing responses is essential for successful regeneration. Acute liver failure results when the wound healing response fails to launch or if the repair process stalls in the midst of tissue-reconstruction when the liver is repopulated by immature liver cells. Chronically futile regenerative responses promote progressive scarring that leads to cirrhosis.

Liver histology has helped to identify which of the various repair-related cell types is mainly responsible for orchestrating regeneration by revealing that myofibroblast accumulation is rare in steatosis, typical in steatohepatitis, and even more conspicuous in cirrhosis. Myofibroblasts (MF) derived from hepatic stellate cells are known to produce most of the fibrosis that is characteristic of alcohol-induced cirrhosis [4], suggesting that cross-talk between dying hepatocytes and hepatic stellate cells (HSC) guides the regenerative process. Hepatic stellate cells are a type of tissue-resident pericyte with characteristics of mesenchymal stem cells, including high plasticity that enables reversible acquisition of myofibroblastic features [5–7]. As discussed subsequently, animal studies demonstrated that transient accumulation of myofibroblasts is necessary for effective liver regeneration while sustained myofibroblast accumulation causes cirrhosis. Evidence linking excessive accumulation of MF-HSC with defective liver repair raises the intriguing possibility that mechanisms that regulate fate decisions in hepatic stellate cells determine the outcomes of alcoholic steatohepatitis. This presentation summarizes data which prove that Hedgehog, a developmental morphogenic signaling pathway, regulates adult stellate cell fate and which demonstrate that dysregulated Hedgehog pathway activity promotes the pathogenesis of acute alcoholic hepatitis and alcohol-induced cirrhosis.

Hedgehog (Hh) regulates tissue construction during fetal development by controlling fate decisions (e.g., proliferation, migration, differentiation, viability) in stem/progenitor cells [8]. Hh signaling is highly conserved across evolution and can be activated in Hh-responsive cells via both paracrine and autocrine mechanisms. In developing fly larvae, for example, epithelial cells produce Hh ligand which interacts

with Hh receptors (dubbed Patched) on Hh-responsive stromal cells to activate the co-receptor (Smoothened) and promote intracellular signaling cascades that culminate in the nuclear localization of the Hh-regulated transcription factor, Glioma-like (Gli). When Hh ligands are absent, Patch represses Smoothened activity and Gli is degraded. Binding of Hh ligand to Patch alleviates its repressive actions on Smoothened and permits Gli accumulation. Gli activity controls the expression of multiple genes that modulate fly morphogenesis**.** The pathway is heavily regulated by lipids: lipids influence ligand availability, ligand-receptor interaction, and Smoothened (Smo) activation [9–12]. Mammals have three Hh ligands (Sonic hedgehog, Shh, Indian hedgehog, Ihh, and Desert hedgehog, Dhh), two Hh receptors (Patched 1, Patched 2) and three Gli transcription factors (Gli1, Gli2, and Gli3), as well as several accessory molecules that regulate pathway activity at various steps in the activation process. In all species, the Hedgehog pathway regulates its own activity by directly controlling expression of pathway components (e.g., Patch, Gli1) and other factors (Hh inhibitory protein, Hhip) that modulate Hedgehog signal transduction. Hedgehog pathway activity, in turn, also regulates (and is regulated by) other important morphogenic pathways, including Notch, Wnt, TGFb, and Hippo-Yap. Like dysregulation of these other pathways, Hedgehog pathway dysregulation has long been known to occur in various types of cancer [13, 14]. However, its role in adult tissue repair has been recognized relatively recently.

Our studies in adult Patched-reporter mice were the first to demonstrate that the Hedgehog pathway is active adult liver by identifying Hh signaling in resident subpopulations of pericytes (a.k.a., hepatic stellate cells) [5]. A decade later, studies in Gli-reporter mice confirmed this finding and provided additional evidence that resident pericyte populations in multiple tissues (e.g., kidney, skin, lung, pancreas) could be identified by their Hh pathway activity [6]. Previous research had already proven that Hh activation stimulated hepatic stellate cells (HSC) to become more myofibroblastic, proliferative and fibrogenic [5, 15, 16]. Further, this work had shown that inhibiting Hh signaling permitted MF-HSC to re-acquire a less myofibroblastic, more quiescent phenotype reminiscent of most of the HSC in healthy adult livers. In addition to validating the earlier findings, a number of recent studies confirmed that inhibiting Hh signaling improved fibrosis in various mouse models of organ fibrosis, including liver cirrhosis [17–20]. Hence, there is now general consensus that the Hh pathway controls the accumulation of MF and fibrosis in many adult tissues, including liver, identifying this pathway as a therapeutic target in alcoholic cirrhosis.

The role of Hh pathway dysregulation in acute liver failure, including that caused by acute alcoholic hepatitis is less-studied. Our research in mouse models has proven that the Hedgehog pathway is abruptly activated, and then subsides, as adult livers regenerates to fully recover functional liver mass after an acute 70% (partial) hepatectomy (PH) [21, 22]. Importantly, effective regeneration also involves transient expansion of hepatic myofibroblast populations and both processes are abrogated by conditional disruption of the Hh signaling pathway in MF-HSC [22]. Rather than regenerating, MF-depleted livers become progressively necrotic. These findings indicate that *transient* expansion of Hh-dependent liver MF populations is

necessary to replace resected hepatocytes so that the liver can regenerate. More recent research from our lab has revealed that this effective regenerative process also involves *transient* de-differentiation of some of the hepatocytes in the liver remnant via mechanisms that are orchestrated by autocrine/paracrine activation of the Hedgehog and Hippo-Yap signaling pathways [23]. This is intriguing because experimental manipulations that cause sustained Yap activation in hepatocytes have been reported to drive hepatocytes to de-differentiate to a stem-like state in which mature hepatocyte functions are lost and liver fibrosis eventually accrues [24–28]. Progenitors and MF accumulate in livers of patients with severe acute alcoholic hepatitis and our group reported that this process parallels the level of hepatic Hh pathway activity and correlates with clinical predictors of acute mortality [29]. We also showed that mice that are genetically predisposed to excessive Hedgehog pathway activity develop worse steatohepatitis than wild type mice that are able to regulate the Hedgehog pathway appropriately during liver injury [30].

Transcriptomic analysis of liver biopsies from patients with severe acute alcoholic hepatitis demonstrated dramatic enrichment with stem/progenitor cell markers [31], suggesting that this condition might reflect dysregulated repair of alcohol-induced liver injury. One of the over-expressed progenitor cell markers, Fn14, encodes a TNF superfamily receptor member that engages the pro-inflammatory cytokine, Tweak (TNF weak-apoptosis inducing factor) [32]. Pro-inflammatory cytokines are increased in severe acute alcoholic hepatitis, suggesting that Tweak-Fn14 signaling would be highly active in this context. Like TNF receptor inhibition, Fn14 deficiency has been reported to inhibit liver regeneration in otherwise healthy mice subjected to acute toxin-induced liver injury or PH [33]. Failed regeneration associated with severely reduced outgrowth of progenitors, suggesting that Tweak-Fn14 signaling normally promotes accumulation of progenitors that eventually become replacement hepatocytes to permit recovery from liver injury. Subsequent studies of explanted livers from patients undergoing liver transplantation for severe acute alcoholic hepatitis support this concept by revealing that excessive Fn14 expression is accompanied by marked expansion of liver progenitor populations [34]. While progenitor accumulation is necessary for eventual recovery from liver damage, the resultant relative deficiency of mature hepatocytes in livers with high pro-inflammatory activity would be expected to compromise liver-specific functions in the short-term and promote acute liver failure. Hence, clinically severe acute alcoholic hepatitis might have occurred because regeneration stalled when the liver was repopulated by immature liver cells.

The possibility that the liver acutely fails in acute alcoholic hepatitis because it has become repopulated by immature hepatocytes has therapeutic implications. Namely, it suggests that mechanisms that promote progenitor accumulation are overly-active and hence, interventions that constrain those processes might be beneficial. As discussed previously, work in animal models has demonstrated that increased Hedgehog signaling, Yap activation, and Tweak-Fn14 induction play critical roles in expanding progenitor populations during liver repair. Pharmacologic agents that are able to inhibit each of these targets are already available [35–37], but none have been tested as treatments for acute liver failure due to alcoholic hepatitis

or other etiologies. It has not yet been possible to model human severe acute alcoholic hepatitis in rodents and thus, the preclinical research would need to test these agents in other types of acute liver failure. If benefits were observed, pilot studies in patients could be considered. The available data also underscore the importance of further research to better delineate the fundamental mechanisms that control liver cell plasticity. There is growing evidence that cellular reprogramming is regulated epigenetically and those mechanisms seem to be influenced by age, gender, and genetic factors [38–40], suggesting that it might be possible to profile individuals who are at particularly high risk for bad outcomes of alcohol-induced steatohepatitis.

11.1 Summary

Alcohol-induced steatohepatitis is a dynamic process. Although it can regress to steatosis or smolder at sub-clinical levels for decades, it also increases the risk for both clinically severe acute alcoholic hepatitis and cirrhosis. The mechanisms that control the outcomes of steatohepatitis are poorly understood but emerging evidence suggests that some of the heterogeneity might reflect differences in processes that control liver cell plasticity. Morphogenic signaling pathways that control cell fate decisions during fetal development, such as the Hedgehog pathway, become reactivated to resume similar functions in injured adult livers. Overly-exuberant activation of these pathways correlates with dysregulated liver repair and increases short- and long-term mortality by promoting acute alcoholic hepatitis or cirrhosis. Manipulating signaling via these pathways may be a novel therapeutic approach to optimize liver cell reprogramming and thus, prevent defective regenerative responses that cause acute and chronic liver failure.

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Chapter 12
Suppressed Fat Mobilization Due to PNPLA3 rs738409 -Associated Liver Damage in Heavy Drinkers: The Liver Damage Feedback Hypothesis

Vanessa Rausch and Sebastian Mueller

Abstract PNPLA3 variant rs738409 has been identified as important progression factor in patients with ALD and NAFLD, the most common liver diseases worldwide. These findings point towards similarities between metabolism of alcohol and fat with regard to the PNPLA3 gene. However, despite many efforts, neither the mechanisms of PNPLA3-related liver damage nor the physiological role of PNPLA3 are fully understood. Based on a large monocentric cohort of Caucasian heavy drinkers we could recently provide evidence that PNPLA3 GG primarily correlated with signs of liver damage (steatohepatitis, ballooning) but less with steatosis. Moreover, upon alcohol withdrawal, PNPLA3 GG carriers showed a delayed inflammation-associated resolution of liver stiffness. In line with the histological findings, hepatic fat content as quantified by CAP (controlled attenuation parameter) did not depend on PNPLA3 status and decreased equally in all genotypes by ca. 30 dB/m during alcohol withdrawal. Preliminary additional analysis from this large cohort indicates that PNPLA3 GG carriers (8.2%) drink significantly less high percentage beverages (23% vs 55%, $p < 0.001$) but show no metabolic phenotype such as increased weight, BMI or diabetes. On the molecular level, key molecules, important for lipolysis and flow of free fatty acids to the liver were drastically reduced in G carriers. These included the liver-synthesized serum ApoA1, the LD-associated protein perilipin5 and the recently identified hepato-protective transcriptional cofactor transducin beta-like-related 1 (TBLR1). Based on these findings, we here introduce the liver damage feedback hypothesis. Accordingly,

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PNPLA3-mediated liver damage (e.g. by enhanced metabolic activity) suppresses the mobilization of fat towards the liver at various levels (reduced serum lipid flux to the liver and fat mobilization from peripheric adipose tissues, suppressed hepatocyte fat release and avoidance of high percentage alcohol beverages). Finally, the liver damage feedback hypothesis identifies a novel and central role of liver damage on systemic fat homeostasis, which has not been appreciated so far.

Keywords Liver damage · Fatty liver · Steatosis · Fat mobilization · PNPLA3 · TBLR1 · Perilipin · Lipid droplet · Lipolysis · Diabetes · Alcoholic liver disease · Fatty acids · Obesity

12.1 Introduction

Alcoholic liver disease (ALD) is the most common chronic liver disease in the Western world [1]. ALD encompasses a broad spectrum of partly overlapping disorders ranging from simple steatosis evolving in nearly all drinkers, to severe forms of liver injury, including alcoholic steatohepatitis, fibrosis and cirrhosis. Most patients will eventually die from alcoholic cirrhosis with hepatocellular carcinoma (HCC, 1–2% per year) as the most common complications of cirrhosis. Although the majority (80–90%) of heavy drinkers with an alcohol consumption >80 g per day develop steatosis, only 35% show signs of inflammation and about 8–20% progress to cirrhosis [2]. Thus, only a small number of drinkers develop severe liver disease suggesting the existence of disease modifiers, which may determine an individual's risk for disease progression while heavy alcohol consumption. The underlying mechanisms are complex and still not fully understood, but suggest interactions between polygenic backgrounds and environmental factors as well as drinking habits (pattern and amount of alcohol consumption) and other liver-related comorbidities such as adiposity or hepatitis infection [3, 4].

12.2 Health Statistics of ALD

Chronic alcohol consumption is one of the major risk factors worldwide affecting significantly both mortality and years of life loss (YLL) [5]. In 2012, nearly half of the world's population consumed alcohol with about 3.3 million deaths (5.9% of all global deaths) attributable directly to alcohol (Global Status Report on Alcohol and health, WHO, 2014). Most of the world's population displays a stable five-year trend in recorded alcohol consumption, while in the African region and the South-East Asia region an increase can be noted (WHO, 2014). Although alcohol affects many other organ systems such as the heart, nervous system, pancreas, breast, the liver remains the major target organ of alcohol consumption and more

than 80% of drinkers will ultimately die from liver-related causes. According to the Global Burden of Disease study 2010 alcohol-attributable liver cirrhosis was responsible for nearly half a million deaths (157,000 female and 336,000 male deaths) [6]. Alcohol-attributable liver cancer was responsible for over 80,000 deaths and liver cancer ranked at position 12 and 16 in the actual death statistics [7]. In 2010 ca. one million people died from liver cirrhosis with nearly 50% of those were directly attributed to alcohol. This is a considerable number when comparing for instant with coronary heart disease with about ten million deaths ranking the leading cause of mortality in the global death statistics. In Central Europe, liver cirrhosis even ranks at the fourth position of YLL and hepatocellular carcinoma (HCC) is now the most common fatal complication of patients with alcoholic liver cirrhosis. Taken together, these dramatic numbers of liver-related death due to alcohol generate high interest in the molecular mechanisms and gene-related factors that drive the disease in only a minority of individuals.

12.3 Genetic Confounders: (Role of PNPLA3)

The role of genetic confounders that render the liver sensitivity to alcohol is underlined by various findings (ALD): First, monozygotic twins have a higher risk to develop alcoholic cirrhosis than dizygotic ones [8, 9]. Second, female drinkers are more sensitive to alcohol when exposed to the same amounts of alcohol than males [10] and third, the white men and women of Hispanic origin are at higher risk for developing alcoholic cirrhosis and have the highest mortality rate when compared to non-Hispanics [11]. In the last decades, continued research and increasing knowledge of genetic variations, the availability of new analytical methods and decreasing costs for genetic studies raises the possibilities to search for genetic factors influencing the course of alcohol-induced liver disease progression. Various candidate genes were identified in case control studies comparing allelic and/or genotypic frequencies of certain genetic variants like single nucleotide polymorphisms (SNP) between individuals with a background of alcohol dependence and ALD [12]. Most human sequence variation is attributable to SNPs, which are found in 1% of the population and occur on average every 1.9 kilobases within the human genome. As a consequence, about 1.42 million SNPs are found in the total human genome [13, 14]. A SNP is a variation in a single nucleotide (e.g. A → C) leading to an altered base triplet potentially coding for a different amino acid, thereby eventually changing the genetic code. This is not always the case due to degeneracy of the genetic code. Variations in the coding region of genes can alter the function of the generated protein; if the SNP changes the amino acid sequence of a protein this is called non-synonymous SNPs, whereas synonymous SNPs do not affect the protein sequence. However, most polymorphisms (>99%) are located in non-coding regions of the genome and have no direct known impact on the phenotype of an individual, but may still affect gene splicing or transcription factor binding [15].

12.4 Discovery of the PNPLA3 Polymorphism rs738409 and Its Role in ALD

Due to genome-wide association studies (GWAS) it was possible to analyze relationships between a given phenotype/disease and millions of SNPs in thousands of different individuals [16]. Using this hypothesis-free approach, a GWAS using a map of SNPs found an association between patatin-like phospholipase domain containing 3 (PNPLA3/Adiponutrin) and plasma levels of liver transaminases in 2008 [17]. Another GWAS performed in the same year by Romeo et al. identified a nonsynonymous SNP out of 9229 SNPs, the rs738409 variant, that encodes for an isoleucine to methionine substitution at position 148 (I148M) in the PNPLA3 gene as genetic variant associated with hepatic triglyceride content (steatosis, $P = 5.9x10^{-10}$) and enhanced inflammation ($P = 3.7x10^{-4}$) in 2111 multiethnic participants of the Dallas Heart study. Individuals heterozygous for this allele had higher hepatic fat levels compared to wild-type carriers, whereas possession of two copies of the 148M allele had a multiplicative effect. This allele was most common in Hispanics, the ethnic group that is also most susceptible to NAFLD and remained highly significant after adjusting for BMI, diabetes mellitus, alcohol abuses as well as global and local ancestry [18, 19]. These findings were quickly confirmed by several population based-studies around the world [20].

In addition, Sookoian et al. showed that this variant also predisposes towards all stages of liver damage starting from simple steatosis to steatohepatitis and progressive fibrosis elevated by histological assessment of liver biopsies [21]. Singal et al. even demonstrated in a meta-analysis that PNPLA3 is associated with an increased risk of advanced fibrosis among patients with different underlying liver disease and is an independent risk factor for hepatocellular carcinoma (HCC) in patients with nonalcoholic steatohepatitis or alcohol-related cirrhosis [22].

Because of the similarities between NAFLD and ALD, these findings stimulated similar genetic analysis in patients with ALD. Tian and coworkers confirmed an association between PNPLA3 and steatohepatitis to cirrhosis in a large Mestizo population (mixed European and Native American ancestry, n = 1221) [23–25]. The study of the genetic variant in the well-characterized Heidelberg ALD cohort of 521 patients (148 females/ 369 males, age range 22–87 years) revealed a significant correlation between the GG genotype with histological steatohepatitis ($r = 0.404$, $P < 0.005$), ballooning ($r = 0.319$, $P < 0.005$) but less with steatosis ($r = 0.264$, $P < 0.05$) (Fig. 12.1) [26]. Furthermore, we found that GG genotype carriers had a shorter duration of alcohol consumption (17.2 vs 18.3 years, Fig. 12.2a) and generally consumed less alcohol than CC type carriers without reaching significance (181 vs. 194 g/day)(Fig. 12.2b and Table 12.1). To our surprise, GG carriers showed also a different drinking behavior; interestingly GG Carriers demonstrated significantly reduced consumption of high percentage beverages such as liquor (23% vs 55%, $P < 0.001$, Fig. 12.2c). This was not due to advanced liver disease or age. No significant differences were recorded for beer and wine intake (data not shown).

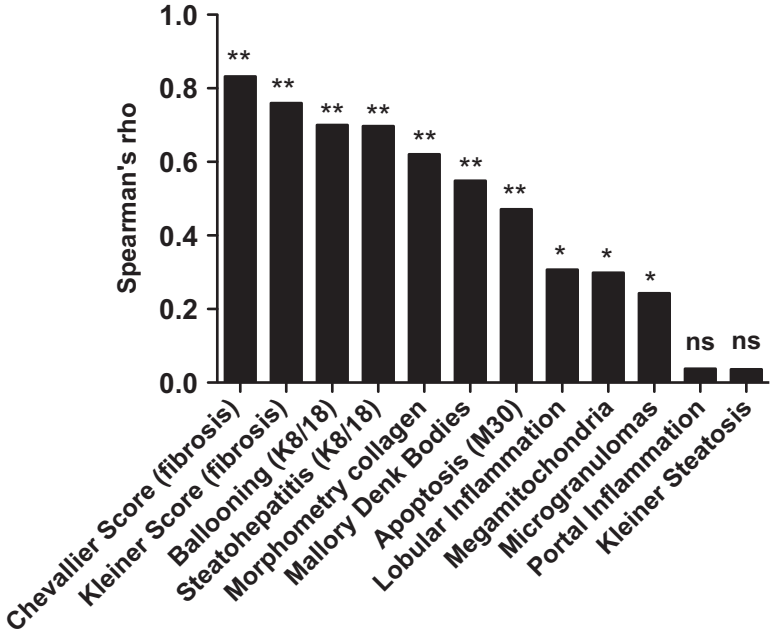

Fig. 12.1 Spearman rank correlation analysis of PNPLA3 GG genotype carriers with histological parameters. * $p < 0.05$; ** $p < 0.01$; ns = not significant

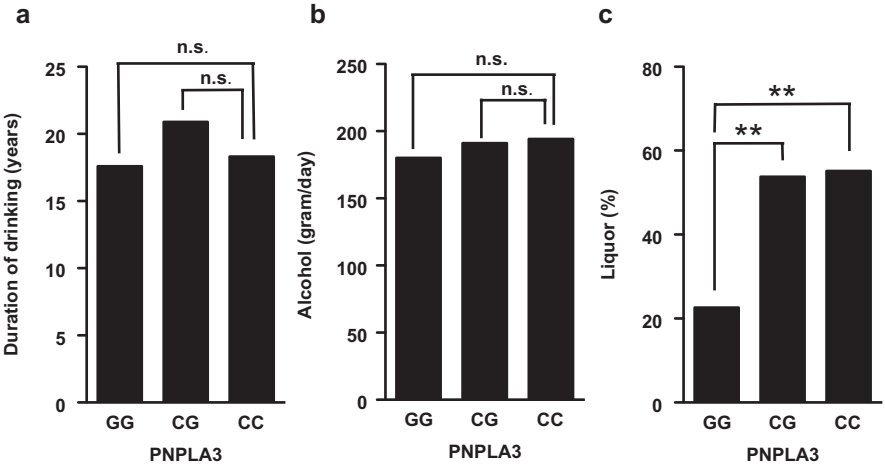

Fig. 12.2 Alcohol drinking characteristics such as (a) duration, (b) daily consumption, (c) liquor for ALD patients depending on the PNPLA3 genotype. GG carrier drink significantly less high percentage alcoholic beverages such as liquor. ** $p < 0.001$

Table 12.1 Characteristics of ALD sub-cohorts based on genotype distribution of rs738409 polymorphism

Parameters	PNPLA3 CC (n = 204)	PNPLA3 CG (n = 274)	PNPLA3 GG (n = 43)	PNPLA3 G (CG + GG) (n = 317)
Demographic characteristics				
Patients (%)	39.2	52.6	8.2	60.8
Age (years)	49.5 ± 11.0	50.7 ± 11.8	50.1 ± 9.7	50.7 ± 11.5
Risk factors				
BMI (kg/m2)	25.4 ± 4.9	25.1 ± 4.5	25.6 ± 3.9	25.2 ± 4.4
H/W ratio	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
Alcohol consumption (g/day)	194 ± 136.1	190.8 ± 146.2	181.2 ± 116.1	189.4 ± 142
Duration (years)	18.3 ± 13.3	20.9 ± 13.1	17.2 ± 14.2	20.4 ± 13.3
Noninvasive parameters				
Hepatic steatosis (0–3, US)	1.8 ± 0.9	2.0 ± 0.8	1.9 ± 0.8	2.0 ± 0.8
Liver stiffness (kPA)	13.1 ± 17.7	17.6 ± 23.0*	17.2 ± 22.2	17.5 ± 22.9+
CAP (dB/m)	288 ± 52	290 ± 56	298 ± 49	291 ± 55
Laboratory parameter				
AST (U/l)	95.2 ± 100.8	102.8 ± 111.4	111.2 ± 116.2	104.0 ± 111.9
ALT (U/l)	66.0 ± 59.4	71.9 ± 93.0	75.1 ± 60.3	72.4 ± 89.1
GGT (U/l)	406.4 ± 572.2	365.9 ± 516.1	525.9 ± 863.0	388.4 ± 578.4
AP (U/l)	105.5 ± 76.2	111.6 ± 75.8	111.5 ± 72.1	111.6 ± 75.2
Bilirubin (mg/dl)	1.2 ± 2.8	1.4 ± 3.0	0.9 ± 1.1	1.3 ± 2.8
Albumin (g/dL)	4.7 ± 4.7	5.3 ± 7.2	4.5 ± 0.5	5.2 ± 6.7
INR	1.4 ± 5.4	1.0 ± 0.4	0.9 ± 0.2	1.0 ± 0.4
Urea	20.6 ± 10.8	24.6 ± 20.2*	20.1 ± 9.9	24.0 ± 19.2+
Creatinine	0.7 ± 0.2	0.7 ± 0.3	0.7 ± 0.2	0.7 ± 0.3
Hemoglobin (g/dl)	14.2 ± 1.8	14.2 ± 2.5	14.6 ± 2.0	14.2 ± 2.4
Platelets (/nl)	216.7 ± 92.7	201.1 ± 80.0*	224.2 ± 91.4	204.5 ± 82.0
Glucose (mg/dL)	112.0 ± 46.2	107.7 ± 28.5	110.7 ± 34.6	108.1 ± 29.3
HbA1C (%)	5.6 ± 1.1	5.6 ± 0.8	5.8 ± 1.3	5.6 ± 0.9
Triglycerides (mg/dL)	190.6 ± 202.2	192.0 ± 205.8	240.9 ± 230.4	198.7 ± 209.6
Cholesterol (mg/dL)	219.9 ± 55.0	213.1 ± 61.1	222.9 ± 53.4	214.4 ± 60.1
HDL cholesterol (mg/dL)	73.2 ± 35.9	71.4 ± 37.6	75.6 ± 37.3	71.9 ± 37.5
LDL cholesterol (mg/dL)	113.5 ± 46.3	112.4 ± 45.5	118.0 ± 44.7	113.0 ± 45.3
Lipase (U/L)	48.5 ± 45.9	75.9 ± 216.5	45.3 ± 26.0	72.0 ± 202.7
Ferritin (ng/ml)	546.1 ± 611.6	599.6 ± 668.3	685.2 ± 708.2	610.8 ± 673.1
CRP (mg/dl)	4.7 ± 11.1	7.1 ± 18.9	6.0 ± 12.0	7.0 ± 18.1

(n = 521). Data are presented as mean +/− SD or in %
*p < 0.05 (CC vs CG); + p < 0.05 (CC vs G)

12.5 Role of PNPLA3 rs738409 in Alcoholic Cirrhosis/Advanced Fibrosis

Tian et al. was the first who reported that PNPLA3 rs738409 GG is strongly associated with alcoholic liver disease, especially with clinically evident alcoholic cirrhosis in Mestizo subjects (unadjusted OR = 2.25, P = 1.7 $x10^{-10}$; ancestry-adjusted OR = 1.79, P = 1.9$x10^{-5}$) [23]. A small study in 266 patients with alcoholic cirrhosis and 182 heavy drinkers from the UK and Australia confirmed the results in Caucasians and demonstrated that carrying of the PNPLA3 rs738409 G allele and GG genotype were also significantly associated with alcoholic cirrhosis (OR = 2.2, P = 2$x10^{-5}$ and OR = 5.57, P = 1.2$x10^{-3}$) [27, 28]. A similar study with a Belgian ALD cohort and corresponding controls (n = 328) showed that the PNPLA3 rs738409 G allele was more frequent in ALD patients and identified as risk factor for cirrhosis (OR = 2.1, P = 0.001) [29]. Furthermore, a large European study performed by Stickel et al. in alcoholics from different centers in Germany also identified an association between PNPLA3 rs738409 GG genotype and liver cirrhosis (OR = 2.79, $P_{genotype}$ = 1.2$x10^{-5}$ and P_{allele} = 1.6$x10^{-6}$) [24]. Falleti as well as Rosendahl et al. confirmed that PNPLA3 rs738409 G allele is more frequent in Caucasians in a study with 483 cirrhotic patients from Italy [30] or 135 patients with alcoholic cirrhosis from Germany and the Netherlands [31]. In addition, Falleti was also the first who identified this SNP as risk factor for HCC development in alcoholic patients with cirrhosis. Only one Asian study in Indians with 120 alcoholics (60 with cirrhosis and 60 without) and 100 controls, confirmed the association between PNPLA3 rs738409 and cirrhosis [32]. The most recent study (n = 387 alcoholics including 206 cirrhotics) performed by Way and Morgan found also an association between GG genotype and cirrhosis with an OR = 2.71 [33]. We also confirmed a weak association in our ALD study cohort and calculated an OR to develop cirrhosis corrected for age, gender and BMI of 1.295 (95% CI 0.787–2.131) for the G genotype [26]. Taken together, all studies including a recent meta-analysis showed an association between PNPLA3 rs738409 G carrier and cirrhosis in patients with alcoholic liver disease (Table 12.2) [34].

12.6 Role of PNPLA3 in Cancer Progression (HCC)

Since PNPLA3 rs738409 was shown to be associated with advanced fibrosis and cirrhosis and this is accompanied in 8–20% of alcoholic cirrhotics with the development of HCC, it was tempting to speculate that this sequence variation might play also a role in liver carcinogenesis. Therefore, case control studies were conducted in ALD patients complicated by HCC or not. Most studies were performed in Europe including Caucasians, except one study performed in 2013 in Japan including

Table 12.2 ORs and 95% CIs of studies analyzing the association between PNPLA3 rs738409 and advanced fibrosis/cirrhosis (adapted from Stickel et al. 2016)

Study (reference)	Type of study	Cases-controls (cirrhotics)	OR or HR (95% CI)	P
Tian et al. 2010 [23]	Case-control	1221 (482)	2.25 (1.74–2.90)	$1.7x10^{-10}$
Seth et al. 2010 [27]	Case-control	448 (266)	5.57 (1.68–18.43)	$1.2x10^{-3}$
Trepo et al. 2011 [29]	Case-control	658 (256)	2.08 (1.15–3.77)	0.02
Stickel et al. 2011 [35]	Case-control	1043 (210)	2.79 (1.55–5.04)	$1.2x10^{-5}$
Falleti et al. 2011 [30]	Case-control	911 (483)	1.76 (1.06–2.92	0.02
Nguyen-Khac et al. 2011 [36]	Case-control	210 (40)	2.5 (1.4–4.4)	0.002
Rosendahl et al. 2012 [31]	Case-control	1510–2781 (135)	2.3 (1.6–3.3)	<0.0001
Dutta et al. 2013 [32]	Case-control	120–100 (60)	2.12 (1.29–3.4)	0.037
Way and Morgan et al. 2013 [33]	Case-control	1106–1058 (212)	2.13 (1.66–2.73)	$1.46x10^{-9}$

OR, odds ratio; HR, Hazardous ratio; CI, confidence interval

patients diagnosed with HCC without confounding virus infections (hepatitis B or C)(n = 104) [37]. Falleti et al. was the first who identified this SNP as risk factor for HCC development in 483 alcoholic patients from Italy where 166 had cirrhosis and calculated an overall OR of 1.76 (95% CI 1.06–2.92, P < 0.05) [30]. Nischalke and Hamza et al. who calculated a 2.8 and 2.6 fold higher HCC risk in patients with ALD confirmed these findings [38, 39]. Trepo et al. even reported a stronger predisposing effect of PNPLA3 genotype and HCC risk in ALD patients [40]. Another study by Guyot et al. also provides data that confirm the influence of PNPLA3 rs738409 G genotype on the occurrence of HCC in patients with alcoholic cirrhosis with a slightly lower OR of 1.72 (95% CI 1.21–2.45, P = 0.002) [41]. A recent meta-analysis of individual patient data from candidate gene association studies (GWAS) found that PNPLA3 rs738409 is strongly associated with overall HCC and found that this association was more pronounced in ALD (OR = 2.20, 95% CI 1.80–2.67) than in patients with HCV-related HCC [40]. Two subsequent meta-analyses confirmed this association [22, 42]. To conclude, several case-control studies and recent meta-analyses have confirmed that PNPLA3 rs738409 G allele is associated with an around two fold HCC risk in alcoholic cirrhotics, but also other modifiers are likely to play a role in liver carcinogenesis (Table 12.3).

Table 12.3 ORs or HR and 95% CIs of studies analyzing the association between PNPLA3 rs738409 and HCC development (adapted from Trepo et al. 2016)

Study (reference)	Type of study (n = number of studies in meta-analysis)	Cases (cirrhotics)	OR or HR (95% CI)	P
Falleti et al. 2011 [30]	Case-control	483 (166)	1.76 (1.06–2.92	<0.05
Nischalke et al. 2011 [38]	Case-control	350 (160)	2.83 (1.24–6.42)	0.013
Hamza et al. 2012 [39]	Case-control	304	2,59 (1.29–5.20)	0.007
Trepo et al. 2012 [40]	Case-control	571	4.7 (2.63–8.42	$1.83x10^{-7}$
Guyot et al. 2013 [41]	Prospective	279	1.72 (1.21–2.45)	0.02
Trepo et al. 2014 [43]	Meta-analysis (n = 5)	1374	2.20 (1.80–2.67)	$4.71x10^{-15}$
Nischalke et al. 2014 [44]	Case-control (with replication)	864 (+229)	2.32 (1.36–4.68)	0.00002
Singal et al. 2014 [22]	Meta-analysis (n = 9)	2937	1.4 (1.12–1.75	n.i.
Salameh et al. 2015 [42]	Meta-analysis (n = 4)	1207	2.81 (1.57–5.01)	n.i.
Friedrich et al. 2015 [45]	Retrospective	421	2.4 (1.29–4.46)	0.008
Falleti et al. 2016 [46]	Case-control	226	2.20 (1.03–4.66)	0.039

OR, odds ratio; HR, Hazardous ratio; CI, confidence interval; n.a. not indicated

12.7 Potential Physiological Functions of PNPLA3

So far, the physiological function of PNPLA3 and the effect of the amino acid substitution remain controversial and the structure of PNPLA3 has never been resolved by crystallization or nuclear magnetic resonance spectroscopy [28]. A clear association of the genetic variant between the GG genotype was found with various liver disease stages including steatosis, steatohepatitis (inflammation and ballooning), fibrosis and cancer [26, 28]. PNPLA3 (adiponutrin) is located on chromosome 22 and encodes a 53 kDa protein with 481-aa length that is closely related to PNPLA2 (also called ATGL), the major hormone-sensitive TAG hydrolase of adipose tissue, sharing 56% amino acid identity in the patatin-like domain [47, 48]. The progenitor of this family, patatin, is a major storage protein of potato tubers but also involved in lipolysis of fatty acids. Structural analysis demonstrated, that the mutation has no influence on the catalytic center but the substrate-binding groove, thereby possibly blocking the access of substrates to the catalytic center [49]. In humans, PNPLA3 is expressed in adipocytes, hepatocytes and hepatic stellate cells [50–53]. PNPLA3 is localized on membranes suggesting an involvement in receptor-like interactions with extracellular signals [54], but also associated with the endoplasmatic reticulum

and lipid droplets (LD) most likely due to the Brummer box [55]. Lipid droplets are the main organelles responsible for neutral lipid storage (primarily TAG and cholesteryl ester) and hydrolysis. Studies demonstrated an upregulation during adipocyte differentiation, and a response to fasting and feeding cycles pointing towards a role in regulation of energy mobilization and lipid storage in adipose tissue and the liver [56]. Furthermore, PNPLA3 mRNA expression was increased in subcutaneous and visceral adipose tissue of obese subjects [57].

12.7.1 Enzyme Function

Three independent *ex vivo* studies demonstrated that the isolated PNPLA3 protein has an enzymatic activity on triglycerides (TG) by using radiolabeled triolein and measuring the release of oleic acid. In two studies they used SF-9 insect cells and one study examined the enzyme function in a yeast system (*Pichia pastoris*) [49, 58, 59]. Although the enzyme showed a predominant lipase activity with oleic acid as major substrate which was also confirmed in Huh7 cells [60], Pingitore et al. found a mild lysophosphatidic acid acyltransferase activity (LPAAT). In addition, Jenkins et al. also described an acylglycerol transacylase activity for PNPLA3. In sum, all three papers demonstrated membrane localization and that the I148M mutation results in a loss of function mutation.

Most *in vitro* studies are performed in immortalized hepatoma cell lines, since this protein is highly expressed in the liver. In these studies PNPLA3 seems to possess triacyl-hydrolase activity on TG embedded in lipid droplets in Huh7 cells and the overexpression of the I148M isoform led to a marked reduction of this enzyme activity when compared with wild type PNPLA3 pointing also towards a loss-of-function mutation and resulting in an accumulation of TG, but not those newly synthesized and thereby trapping lipids in the liver [52]. Furthermore, it has been proposed that PNPLA3 148 M may promote intracellular lipid accumulation by reducing hepatic very low density lipoprotein (VLDL) synthesis [61]. VLDLs are particles highly enriched in TG and secreted by the liver to provide different lipids for peripheral tissues [62]. Retention of VLDLs in the liver causes increased hepatic fat content [63]. These findings were also confirmed in humans by measuring hepatic VLDL secretion by injection of stable isotypes in different PNPLA3 carriers [28]. Furthermore, studies showed that silencing of the carbohydrate response element-binding protein (ChREBP) abolished the induction of PNPLA3 mRNA by glucose in immortalized human hepatocytes suggesting a glucose dependent regulation of PNPLA3 [64]. Finally, a recent study by Pirazzi et al. suggested a retinyl-palmitate hydrolase activity for PNPLA3 in hepatic stellate cells in humans [53] and demonstrated that I148M mutation is associated with reduced levels of retinol binding protein 4 (RBP4), the major transport protein for vitamin A in the blood [65]. It is known that HSCs lose their

retinol-storing ability while activation and differentiation into myofibroblasts-like cells that secrete collagen thereby contributing to liver fibrosis. However, this indicates a potential novel link between HSCs, retinoid metabolism and PNPLA3 in determining the susceptibility to chronic liver disease whereas the specific role of PNPLA3 in this process remains still unclear.

12.7.2 In Vivo Studies

However, *in vivo* studies using genetically modified mice overexpressing or silencing PNPLA3 (knockout mice models) proved to be difficult, since deletion of PNPLA3 lead to no altered phenotype, suggesting a distinct enzyme function in mice or that other enzymes may compensate for the lack of PNPLA3 activity. The first approach studying PNPLA3 function *in vivo* was performed via adenoviral PNPLA3 overexpression. He et al. observed that overexpression of wild type PNPLA3 in mouse liver showed no obvious phenotype and did not lead to reduced hepatic TG content, while the I148M mutant protein increases liver fat content [52]. This could suggest a gain of function and a lipogenic role for PNPLA3 that is enhanced in the presence of the mutant variant. In addition, two other studies performed in PNPLA3 knockout mice indicated no major role for PNPLA3 in steatosis development [66, 67]. However, studies in transgenic mice overexpressing the sterol regulatory element binding protein-1c (SREBP-1c) showed an upregulation of PNPLA3 mRNA implicating an important link between PNPLA3 and the insulin signaling pathway, since SREBP-1c is a downstream target [68]. Another study performed in high-fat diet fed rats, in which endogenous PNPLA3 was silenced using specific antisense oligonucleotides also pointed towards a possible role of PNPLA3 in insulin signaling [69]. Furthermore, in obese humans PNPLA3 I148M mutation was shown to be associated with an increased risk of type 2 diabetes [70]. A more recent knock-in mouse model used to either overexpress the mouse I148M mutant protein or the S47A variant resulted in hepatic lipid droplets accumulation and steatosis development when mice were fed with a high sucrose diet, indicating a loss of function mutation [71]. Today, this mouse model is the one most closely resembling the human physiologic condition.

12.8 Recent Findings from the Heidelberg Mono–Center Cohort of Heavy Drinkers – The Role of Alcohol Withdrawal

We recently analyzed the role of the PNPLA3 genotype in over 500 Caucasian heavy drinkers admitted for alcohol detoxification. Expression levels of various mRNA transcripts, histology, serum markers and many clinical parameters including

fibrosis (transient elastography, Fibroscan) and steatosis (CAP, controlled attenuation parameter) were performed. The PNPLA3 rs738409 genotype distribution for CC, CG and GG was 39.2%, 52.6% and 8.2%. Mean LS was lowest in CC carriers (13.1 kPa) as compared to CG and GG carriers (17.6 and 17.2 kPa) (Table 12.1). Interestingly, almost no change was observed in CC carriers after alcohol withdrawal (12.0 kPa, LS2) while LS significantly decreased in CG carriers to comparable 12.7 kPa. Despite a longer observation interval of 6.6 days, LS decreased slower in GG most likely due to sustained inflammation/liver injury as reflected by enhanced AST levels (26). Moreover, hepatic fat content (CAP) was only non-significantly increased in GG carriers and it decreased equally in all groups by 30 dB/m (Table 12.1). Like LS, no significant decrease of CAP was seen in GG carriers (26). In summary, LS was highest in G (GG and CG) carriers resolving to baseline levels comparable to the CC genotype after alcohol withdrawal. In contrast, fat content as measured by CAP was almost identical and decreased equally in all groups after alcohol detoxification. In conclusion, in heavy drinkers, PNPLA3 GG primarily correlates with liver damage but not steatosis resulting in a delayed inflammation-associated resolution of LS as reflected by increased AST levels. Consequently, sustained LS elevation could be a major risk factor in PNPLA3 GG carriers.

12.9 Evidence of Suppressed Fat Mobilization in the Heidelberg Cohort

Interestingly, we could identify several lines of evidence that the PNPLA3 GG associated liver damage is associated with reduced fat mobilization to prevent further hepatic fat loading at various levels. First, we detected that the recently discovered transcriptional cofactor transducin beta-like-related 1 (TBLR1), identified as protective factor against hepatic steatosis in the metabolic syndrome was drastically reduced (Fig. 12.3a) and correlated negatively with the GG genotype ($r = -0.537$; $p < 0.01$) (Table 12.4). Its ligand TBL1 was also negatively correlated although not reaching levels of significance. Surprisingly, the C genotype was significantly associated with liver-synthesized ApoA1 ($r = 0.38$; $p < 0.01$, data not shown), which is known to promote fat efflux from adipose tissues. Significantly decreased ApoA1 levels were seen in G carriers (Table 12.5). In addition, analysis of the expression of lipid droplet-associated proteins of the perilipin family that regulate lipid droplet biogenesis, maintenance and degradation in hepatocytes revealed that Plin5 mRNA was markedly reduced in CG carriers and in the G genotype while no significant differences were observed for Plin3, though both perilipins represent exchangeable proteins shuttling between cytoplasm and lipid droplets dependent on functional state of cells (Straub et al. in press). Similar results were found for plin5 on protein level, where Plin5 was less recruited to lipid droplets in CG and in GG carriers showing a rather diffuse weak staining (Fig. 12.3c and Straub et al. in press). Likewise, Spearman correlation analysis demonstrated a significant negative

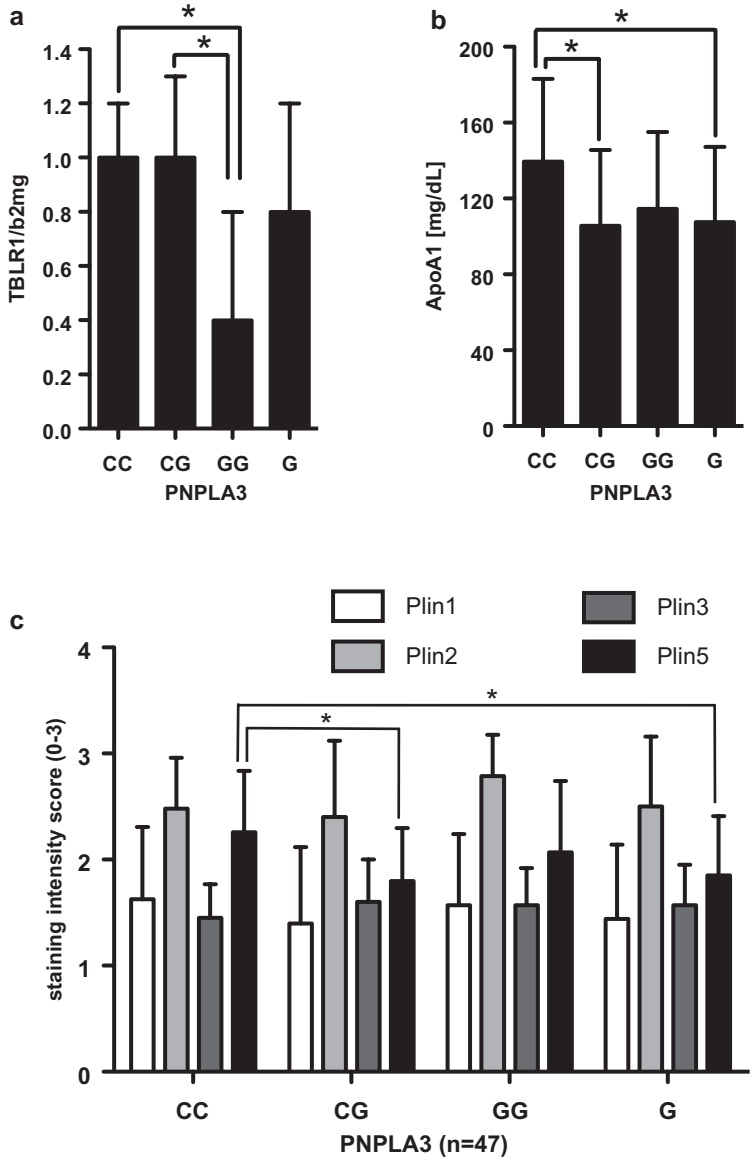

Fig. 12.3 PNPLA3 genotype-dependent expression of (a) TBLR1 mRNA, (b) serum ApoA1 levels and (c) perilipin (plin1, 2, 3 and 5) mRNAs. TBLR1, ApoA1 and plin5 are suppressed in GG carriers. Data are presented as mean+/− SD. * $p < 0.05$. mRNA expression analysis of n = 24 liver biopsy samples

Table 12.4 Spearman rank correlation analysis of PNPLA3 GG genotype carriers with clinical, morphological, histological and molecular parameters

PNPLA3 GG		
Parameter	Method	rho
Ballooning	Histology	0.332**
Microgranulomas	Histology	0.315**
Steatosis (Kleiner score)	Histology	0.235*
ALT (U/L) 2	Serum	0.106*
Triglycerides (mg/dL)	Serum	0.096*
Liver size	Ultrasound	0.089
AST (U/L) 2	Serum	0.087
TBLR1	Liver mRNA	−0.537**
Plin5	Liver mRNA	−0.442*
Portal inflammation	Histology	−0.186
Liquor	Medical history	−0.167**

* $p < 0.05$; ** $p < 0.01$; 1 = before alcohol withdrawal, 2 = after alcohol withdrawal

Table 12.5 Lipid stores and parameters in PNPLA3 genotypes

Compartment	Parameter	PNPLA3 CC	PNPLA3 CG	PNPLA3 GG
Peripheral fat	BMI (kg/m2)	25.4 ± 4.9	25.1 ± 4.5	25.6 ± 3.9
Serum lipids	Triglycerides (mg/dL)	190.6 ± 202.2	192.0 ± 205.8	240.9 ± 230.4
	Cholesterol (mg/dL)	219.9 ± 55	213.1 ± 61.1	222.9 ± 53.4
Lipid trafficking	ApoA1 (mg/dL)	139.4 ± 43	**105.6 ± 39.2***	**114.5 ± 37.6***
	MLDP/Plin5	4.4 ± 1.3	4.0 ± 2.0	**2.0 ± 0.2***
	TBLR1	1.0 ± 0.2	1.0 ± 0.3	**0.4 ± 0.4***
Liver fat	Steatosis (US)	1.8 ± 0.9	2.0 ± 0.8	1.9 ± 0.8
	Steatosis (histology)	1.9 ± 1.0	1.9 ± 0.1	**2.6 ± 0.5***
	Steatosis (CAP)	288 ± 52	290 ± 56	298 ± 49
Liver damage	AST (U/L) (bw)	95.2 ± 100.8	102.8 ± 111.4	111.2 ± 116.2
	AST (U/L) (aw)	47.8 ± 32.8	52.6 ± 45.9	**82.8 ± 87.8***
	Ballooning (histology)	0.7 ± 0.7	0.8 ± 0.8	**1.4 ± 0.5***
	Steatohepatitis (histology)	1.1 ± 0.8	1.1 ± 0.7	**2.0 ± 0.0***

Data are presented as mean +/− SD
*p < 0.05 (CC vs CG or CC vs GG)

correlation between plin5 and GG type ($r = -0.443$; $p < 0.05$) (Table 12.4). In line with our findings, a recent study showed that PNPLA3 I148M is associated with lower *de novo* lipogenesis and a reduction of liver SREBP-1c mRNA levels, despite increased hepatic fat content [72]. This may point towards a compensatory mechanism of hepatic fat increase in subjects with the I148M allele. Taken together, our findings on liver-expressed ApoA1, TBLR1 and plin5 point towards reduced hepatocellular lipolysis and fatty acid mobilization in the liver in PNPLA3 GG carriers. Furthermore, the data on decreased plin5 levels in PNPLA3 G genotype could provide a novel link between PNPLA3 pathophysiology on lipid droplet-formation.

12.10 Reduced Fat Mobilization Due to PNPLA3-Associated Liver Damage: The Liver Damage Feedback Hypothesis

Based on the observations above we propose the following hypothesis named liver damage feedback hypothesis: In heavy drinkers PNPLA3 G seems to predispose to primary liver damage leading to reduced backflow of fatty acids to the liver via ApoA1 (serum lipid trafficking) and inhibited fatty acid utilization via TBLR1 (hepatocellular lipolysis signaling) and plin5 (regulation of fat flux to mitochondria) (Fig. 12.4), which may be a compensatory negative feedback loop in G carriers to prevent further hepatic lipid loading. To sum up, the naturally occurring mutation in PNPLA3 leads to inflammation and fibrosis development combined with disturbed intrahepatic lipid remodeling via suppressed lipolysis and fatty acid mobilization. Due to the similarities between NAFLD and ALD pathophysiology, and the fact that alcohol directly converts into fat, it is reasonable to assume, that the latter is the real problem. Since the variant of PNPLA3 is not directly linked with insulin sensitivity or BMI and does not affect related metabolic disorders such as dyslipidemia or type 2 diabetes [73], and we cannot detect a significant difference in liver fat content in heavy drinking G carriers (CAP), we hypothesize that they might have a facilitated/accelerated fatty acid oxidation which in addition increases liver disease progression. This facilitated FA/TG hydrolysis might be under the control of Plin5 acting as a lipolytic barrier to prevent uncontrolled TG mobilization/shuttling and FA oxidation in the mitochondria [74, 75]. It remains unclear whether the I148M substitution independently of fibrosis directly causes steatosis, lipotoxicity, or both and how it influences hepatocarcinogenesis.

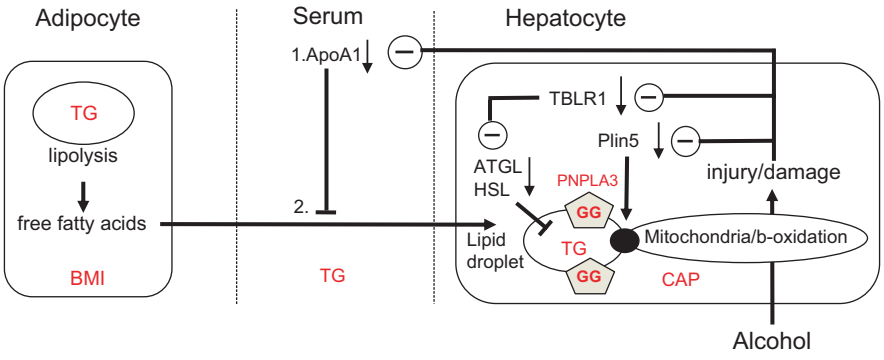

Fig. 12.4 The liver damage feedback hypothesis: PNPLA3 GG seems to cause primary liver damage leading to reduced backflow of fatty acids to the liver via ApoA1 (serum lipid trafficking) and inhibited FA utilization via TBLR1 (hepatocellular lipolysis signaling) and plin5 (regulation of fat flux to mitochondria)

12.11 Other Genetic Variations that Promote ALD

In 2015, a GWAS performed by Buch et al. in patients with ALD comparing 1426 heavy drinkers without indicated liver injury to 712 patients with alcohol-induced cirrhosis in Europeans with a subsequent validation in two independent European cohorts (922 controls and 1148 cases) reported that TM6SF2 ($P = 7.89x10^{-10}$) and MBOAT7 ($P = 1.03x10^{-9}$) are important risk loci for alcohol-related cirrhosis and confirmed the role of rs738409 in PNPLA3 ($P = 1.54x10^{-48}$) at a genome-wide level of significance. These three independent loci are all involved in lipid metabolism, suggesting that lipid turnover is important in the pathogenesis of alcohol-related cirrhosis [76]. Furthermore, Falleti et al. identified TM6SF2 in conjunction with PNPLA3 as potential genetic risk factors for developing HCC in alcohol-related cirrhosis ($P = 0.0007$) [46]. Overall, it is likely that in future more GWAS studies are performed leading to the identification of additional variants robustly associated with alcohol-induced liver damage.

12.12 Conclusion and Future Perspectives

In the search for genetic risk factors rendering man more susceptible for-alcohol-induced liver disease, PNPLA3 was the first locus to be reproducibly and strongly associated with steatosis, fibrosis/cirrhosis in various liver diseases with different etiologies including NAFLD, ALD, and CHC and even HCC. The various studies on PNPLA3 allow the conclusion that PNPLA3 rs738409 GG carrier represent a subpopulation of high-risk subjects susceptible to develop ALD and cirrhosis. Genetic association studies highlighted a role of PNPLA3 in fat metabolism and a major impact on the development of liver disease but lipodomic analyses have not been performed, yet. If lipid turnover by the mitochondria is disturbed or another mechanism/pathway is influenced by the I148M variant still remains an open question and definitely needs further investigations.

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Chapter 13
Aldo-Keto Reductases: Multifunctional Proteins as Therapeutic Targets in Diabetes and Inflammatory Disease

Kun-Che Chang and J. Mark Petrash

Abstract Aldose reductase (AR) is an NADPH-dependent aldo-keto reductase that has been shown to be involved in the pathogenesis of several blinding diseases such as uveitis, diabetic retinopathy (DR) and cataract. However, possible mechanisms linking the action of AR to these diseases are not well understood. As DR and cataract are among the leading causes of blindness in the world, there is an urgent need to explore therapeutic strategies to prevent or delay their onset. Studies with AR inhibitors and gene-targeted mice have demonstrated that the action of AR is also linked to cancer onset and progression. In this review we examine possible mechanisms that relate AR to molecular signaling cascades and thus explain why AR inhibition is an effective strategy against colon cancer as well as diseases of the eye such as uveitis, cataract, and retinopathy.

Keywords Aldose reductase · Inflammation · Diabetes · Cancer · Inhibitor · Cataract · Retinopathy · Nephropathy · Neuropathy

13.1 Introduction

Aldo-keto reductases (AKRs) are a superfamily of enzymes involved in phase 1 metabolism of carbonyl substrates such as sugars, lipid aldehydes, keto-steroids and keto-prostaglandins [1–4]. The AKR superfamily contains 16 families (AKR1–16) [5] based on their high sequence similarity and common protein fold structure [AKR website: http://www.med.upenn.edu/akr/]. Enzymes within this family share many catalytic and structural properties. As a group, AKRs are nicotinamide

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Table 13.1 The table for human aldo-keto reductase family 1B

Gene	Protein	Also known as	Chromosomal localization
AKR1B1	Aldose reductase	AR, ALR2	7q33
AKR1B10	Small intestine-like aldose reductase	HSIR, ALRl	7q33
AKR1B15	Aldo-keto reductase family 1, member B15	AKR1B10L	7q33

adenine dinucleotide (phosphate) (NAD(P)H)-dependent oxidoreductases and are expressed as 34–37 kDa polypeptides [6].

The AKR family 1 includes: AKR1A (aldehyde reductases) [7], AKR1B (aldose reductases) [8], AKR1C (hydroxysteroid dehydrogenases) [9], AKR1D (steroid 5β-reductases) [9], and AKR1E (1,5-anhydro-D-fructose reductase) [10]. Among enzymes in the AKR family 1, AKR1B is the most well-studied with well over 6000 reports published in PubMed (as of September 2016). Three AKR1B subfamily enzymes include AKR1B1 (human aldose reductase, HAR), AKR1B10 (human small intestine-like aldose reductase, HSIR) and AKR1B15 that are all encoded by genes localized to chromosome 7 [11] see Table 13.1. Since it is so well-studied among aldo-keto reductases, this review will focus primarily on recent studies on the role of aldose reductase (AR, AKR1B1) in human health, cancer, and ocular disease.

13.2 Function of AR in Cellular Responses

13.2.1 Inflammation

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a transcription factor that controls gene expression affecting cellular processes such as cell cycle regulation, apoptosis [12], and activation of genes involved with inflammation [13]. In unstimulated cells, NF-κB is localized mainly in the cytoplasm [14]. Following cellular activation, NF-κB translocates into the nucleus and becomes acetylated by histone acetyltransferase (HATs) including CREB-binding protein (CBP) or its homolog p300 [15, 16]. Studies have shown that acetylation enhances both DNA-binding ability and transcriptional activity of NF-κB [17]. Removal of acetyl groups, catalyzed by a family of deacetylating proteins, can also influence the activity of transcription factors. SIRT1 (silent mating type information regulation 2 homolog) 1, the sirtuin 1 protein in mammals, is an NAD^+-dependent deacetylase that has been known to inhibit NF-κB transcription signaling by removing acetylation from its subunit RelA/p65 at lysine 310 [18]. Therefore, activity of SIRT1 plays a crucial role in regulating inflammatory responses by influencing the acetylation, and thus the activation state of NF-κB, the key transcription factor controlling expression of proinflammatory genes.

Pioneering studies by Ramana and Srivastava and their colleagues demonstrated a possible connection between AR and NF-κB activation when they showed that ARIs suppress the endotoxin-induced activation of NF-κB in macrophages [19]. Ramana and Srivastava have studied the intersection of AR and NF-κB by focusing on the role of AR in the NADPH-dependent detoxification of reactive aldehydes [20, 21]. AR has been shown to play a role in the reduction of toxic aldehydes such as 4-hydroxy-*trans*-2-nonenal (HNE) and its glutathione adducts (GS-HNE). Under oxidative stress conditions characterized by increased levels of lipid peroxidation, AR converts HNE and GS-NHE to 1,4-dihydroxynonene (DHN) and GS-DHN, respectively [22]. GS-DHN is the predominant metabolite derived from HNE due to the high reactivity of the aldehyde with glutathione. GS-DHN is a potent activator of the phospholipase C (PLC)/protein kinase C (PKC)/NF-κB pathway [23, 24]. Therefore, several studies indicated that AR can play an important role in the activation of the PLC/PKC/NF-κB cascade: AR inhibition suppresses these signaling events [23, 25–27]. In ocular models of endotoxin-induced uveitis, injection of LPS induces NF-κB activation in the anterior or posterior chambers of the eye [28, 29]. Clinically, topical corticosteroids, which act by suppressing NF-κB signaling [30], represent the primary treatment strategy for patients with noninfectious anterior uveitis [31, 32].

13.2.2 Intersection of Glucose Metabolism and Inflammatory Signaling

Hyperacetylation of NF-κB is well known to drive expression of inflammatory signaling genes [33]. Among many factors, high glucose has been shown to cause increased acetylation of NF-κB [34]. We hypothesize that the polyol pathway may provide a functional link between glucose metabolism, protein acetylation, and NF-κB activation. In the first step of the polyol pathway, AR catalyzes the NADPH-dependent conversion of glucose to sorbitol, which is then converted to fructose by the NAD^+-dependent sorbitol dehydrogenase (SDH) [35]. Thus, accelerated glucose flux through the polyol pathway results in a reduction in the redox ratio of NAD^+/NADH. Consequently, lower levels of NAD^+ could be expected to attenuate the NAD^+-dependent deacetylase activity of sirtuin-1 (Sirt-1), resulting in higher levels of NF-κB acetylation and therefore higher activity of Sirt-1 as an activator of inflammatory gene transcription. Thus, the imbalance between NAD^+/NADH provides a plausible linkage between polyol pathway activation and accumulation of acetyl-NF-κB, leading to an inflammatory phenotype (Fig. 13.1). We hypothesize that blockade of the poylol pathway by inhibition of either AR or SDH would prevent the high glucose-induced redox imbalance and thereby support the higher deacetylase activity of sirtulin 1. This would result in reduced levels of activated NF-κB and thereby lower expression of inflammatory genes.

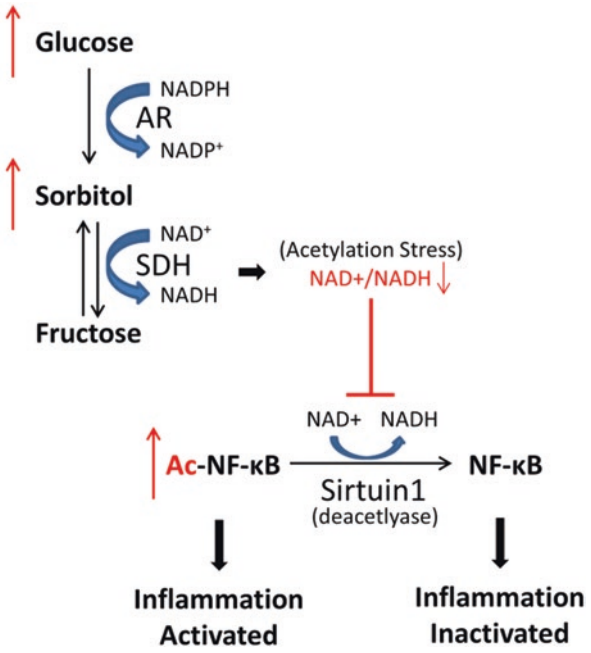

Fig. 13.1 Linkage between polyol pathway activation and induction of inflammatory signaling. Glucose metabolism through the polyol pathway results in a reduced NAD^+/NADH ratio, which lowers the ability of Sirt1 to deacetylate NF-κB

Our studies are in concordance with those mentioned previously by Ramana and Srivastava [36–38], demonstrating that AR inhibition prevents endotoxin-induced inflammation in the eye [39, 40]. However, further studies will be needed to elucidate whether AR inhibition protects against the inflammatory response through its control of lipid derived aldehydes or alternatively through its influence on NAD^+/NADH ratios and acetylation status of transcription factors such as NF-κB. It is also possible that AR is involved in the inflammatory response via both pathways.

13.2.3 Oxidative Stress

Hyperglycemia is a leading cause of oxidative stress in diabetic organs such as heart, kidney and eye. Pathways of hyperglycemia-induced oxidative stress include polyol pathway, mitochondrial electron transport system, protein kinase C (PKC) and advanced glycation end products (AGEs) (Fig. 13.2) [23]. During hyperglycemia, increased flux of AR-mediated reduction of glucose into sorbitol in a NADPH-dependent reaction was observed [41, 42]. NADPH plays reductive roles in metabolic steps, such as detoxification of reactive oxygen species (ROS) by the glutathione reductase/glutathione peroxidase system [43]. Therefore, excessive utilization of NADPH by the polyol pathway could compromise the ability of cells to protect themselves from oxidative stress.

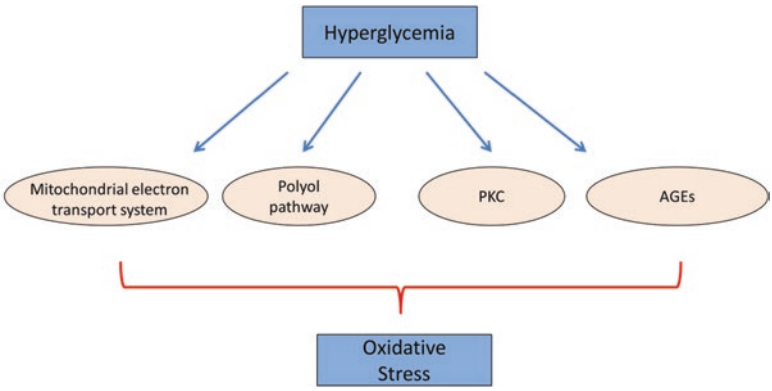

Fig. 13.2 Hyperglycemia-induced oxidative stress is contributed by mitochondrial electron transport system, polyol pathway, PKC and AGEs

In the secondary part of the polyol pathway, sorbitol is converted to fructose by SDH resulting in decreased ratio of NAD^+/NADH. Elevation of cytosolic NADH/NAD^+ ratio leads to induction of ROS via mitochondrial NADH dependent pathway [44]. Increased NADH could also enhance the synthesis of diacylglycerol (DAG), which activates PKC and subsequently induces oxidative stress via PKC-dependent activation of NAD(P)H oxidase [45].

In addition to pyridine cofactor imbalances, AGEs resulting from hyperglycemia also contribute to oxidative stress [46]. AGEs are formed by nonenzymatic glycation reactions involving addition of a carbohydrate to a protein under high glucose conditions typical of diabetic individuals [46]. A study using glucose and fructose in comparison of the ability of forming AGEs showed that fructose forms AGE-BSA much faster than glucose [47]. This observation indicates that elevation of fructose from increased flux of the polyol pathway is another contributor for oxidative stress. Collectively, reduction of the ratio of NADPH/$NADP^+$ and NAD^+/NADH, and induction of AGEs are the major causes of oxidative stress in hyperglycemic environment (Fig. 13.3).

13.3 AR and Complications of Diabetes and Chronic Hyperglycemia

Many studies implicate AR in inflammatory responses in immune cells [36–40], in heart [48], in kidney [49, 50] and in the eye [39, 51–53]. Additionally, AR is a major factor that causes a variety of diabetic complications such as autonomic neuropathy [54–58], ischemic myocardial injury [59–68] cardiomyopathy [48, 69, 70], nephropathy [71–73], cataract [74–79] and retinopathy [80–84] see Table 13.2. Pharmacological inhibition of AR or genetic deficiency in animal models with

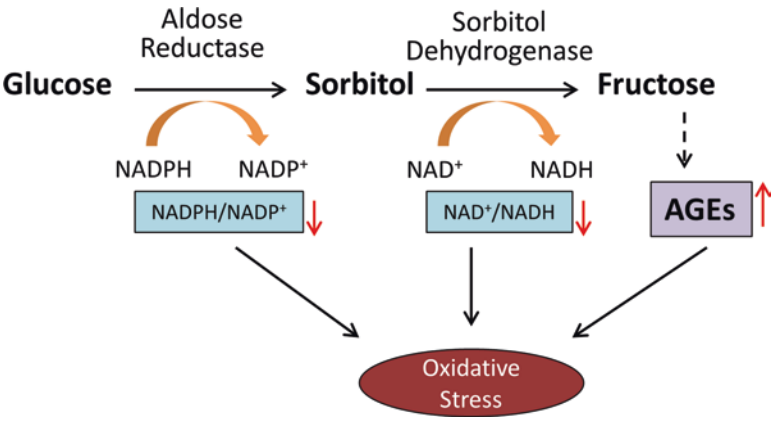

Fig. 13.3 Increased flux of polyol pathway initiates oxidative stress by reducing the ratio of NADPH/NADP+ and NAD+/NADH, and inducing AGEs production

Table 13.2 The table for the role of AKR1B1 in health and disease

Organ	Associated disease
Heart	*Myocardial ischemic injury*
	Cardiomyopathy
Kidney	*Diabeitc Nephropathy*
	Acute Kidney Injury
Nerve	*Diabetic peripheral neuropathy*
	Cardiac autonomic neuropathy
Eye	*Uveitis*
	Diabetic cataract
	Diabetic retinopathy
	Posterior Capsular Opacification

targeted disruption of the AR gene (AR knock out) brings about protection against these complications of diabetes and therefore provide a valuable tool for investigating pathogenesis caused by endotoxin or diabetic hyperglycemia. In this review, we will focus on AR inhibitors' effects on the major complications of diabetes mellitus.

13.3.1 Heart

13.3.1.1 Myocardial Ischemic Injury

Myocardial ischemia is a heart disease caused by lack of oxygen to cardiac muscle, usually due to blockage of blood vessels. Diabetic patients have high incidence of cardiovascular disease and myocardial infarction [85, 86]. In diabetic patients, more

sorbitol accumulation and a decrease of NADPH, from a flux of glucose through the polyol pathway, have been considered a causal factor in cardiac dysfunction [56, 87]. Many AR inhibitors such as Zopolrestat [59–61, 64], Tolrestat [63, 67] and Sorbinil [63, 67] have been reported effectively against myocardial ischemic injury in mice [64], rat [59, 60, 67] and rabbit [61, 63] models in both diabetic and non-diabetic conditions. To further understand the influence of AR in the heart, Ramasamy [62] and Bhatnagar [66] groups conducted experiments to measure AR activity and kinetic properties between normal and ischemic heart. They both found that ischemia increases myocardial AR activity, with higher *Kcat* and *Vmax* due to oxidative stress, without affecting *Km* [62]. In addition, the expression and activity of AR was significantly higher in aged hearts than young ones in a rat model [68]. Treating aged rats with AR inhibitor reduced ischemic injury and improved cardiac function in aged hearts [68]. Therefore, there is still a need for developing AR inhibitors on myocardial ischemia therapy.

13.3.1.2 Cardiomyopathy

Cardiomyopathy is degeneration of the myocardium, which causes severe cardiac failure and arrhythmia [88]. Studies showed that AR activation induces oxidative stress [39, 80] which could further trigger the NF-κB pathway [36, 38, 48]. The NF-κB pathway is involved in inflammatory condition which contributes to cardiovascular dysfunction [89]. Sakamoto and Sugamoto observed the upregulation of AR-like gene in heart of cardiomyopathic rodent [70]. Ramana and colleagues also reported that AR inhibition is capable of preventing endotoxin-induced cardiomyopathy [48]. In addition, AR inhibition is able to prevent acute hyperglycemia-induced cardiac contractile dysfunction by reducing oxidative stress [69]. Taken together, observations above indicate that blockade of NF-κB pathway and oxidative stress through AR suppression could be a therapeutic strategy for preventing cardiomyopathy.

13.3.2 Kidney

13.3.2.1 Nephropathy

Diabetes has high influences in kidney complications and approximately 30% of diabetic patients have diabetic nephropathy, which is a leading cause of kidney failure in US [90, 91]. A strong immunohistochemical staining of AR was found in diabetic individuals, when compared to non-diabetic individuals, indicating that the level of AR is increased in the kidney of diabetics [92]. Supportive studies reported that high glucose in blood or cultured condition induces AR expression that leads to renal sorbitol accumulation [93–95], reactive oxygen species (ROS) production [96, 97] and inflammation [96] in nephropathy kidney or renal cells culture. Thus, a

variety of AR inhibitors had been utilized for treating nephropathy by inhibiting the polyol pathway [73] but showed no influences on kidney weight, body weight or blood glucose [72]. In vivo studies utilizing AR null mice showed that genetic ablation of AR plays a strong protective role in preventing diabetic nephropathy [71]; however, the AR deletion in kidney came with abnormal functioning of the inner medulla [98]. Kidneys from diabetic patients also highly express TGF-β [99], which is an inducer of epithelial-mesenchymal-transition (EMT) [100]. Evidence from human mesangial cells showed that AR inhibition prevents transforming growth factor-beta 1 (TGF-β1)-induced fibronectin expression [101], which is an EMT marker that leads to kidney fibrosis. Another study conducted in renal proximal tubular cells showed that AR inhibition attenuated hyperglycemia-induced fibronectin elevation [93]. Collectively, AR inhibition could be a therapeutic strategy by preventing ROS production and EMT marker expression in patients of nephropathy.

13.3.2.2 Acute Kidney Injury

Acute kidney injury (AKI), also called acute renal failure (ARF), is a rapid onset loss of kidney function that may arise from an intense inflammatory process. In AKI mouse model, endotoxin injection increases the levels of blood urea nitrogen, creatinine and cytokines which cause vacuolar degeneration, apoptosis of renal tubular cells and immune cells infiltration [50]. Pretreatment with Fidarestat, an ARI, was able to ameliorate LPS-induced AKI by reducing inflammation and increase survival rate [49, 50]. The polyol pathway has recently been implicated in ischemia/reperfusion tissue injury. Hindlimb ischemia in mice revealed accumulation of sorbitol and fructose in ischemic muscles accompanying secretion of TNF-α and IL-6 in serum, which led to AKI [49]. Treatment with the AR inhibitor was effective at suppressing inflammatory reactions and renal failure [49]. These results suggest that AR inhibition may be a potential therapeutic strategy for treatment of AKI.

13.3.3 Peripheral Nervous System Disorders

13.3.3.1 Diabetic Peripheral Neuropathy

Diabetic peripheral neuropathy (DPN) is nerve damage that affects the 50% of patients with both Type 1 and Type 2 diabetes [102] and causes loss of sensation in the arms, hands, legs and feet [103]. DPN is considered one of the most painful complications affecting diabetic patients [104]. Patients usually feel painful prickling, burning, electrical, sharp, or jabbing sensation [105]. Assessing sensory nerve conduction velocity (SNCV) and motor nerve conduction velocity (MNCV) allows clinicians to determine the degree of neuronal activity or damage [106]. Measurement of F-wave latency is the most common procedure to diagnose peripheral neuropathy

[107]. The efficacy of treatment on DPN is determined using electrophysiological measurements of three key nerves—median motor, tibial motor, and median sensory nerves. A variety of strategies have been explored for management of DPN including the use of oriental medicine and natural products [108]. Conventional treatments of DPN include the commonly known analgesic drug such as non-steroidal anti-inflammatory drugs (NSAIDs) and opiates [109, 110]. Among the medicine in the management of DPN, tramadol is often used as an analgesic. Tramadol is a semisynthetic opium-derived compound that binds to μ and δ opioid receptors and interferes the re-uptake of serotonin and norepinephrine [111, 112]. However, clinical use of tramadol is not recommended due to the high risk of epileptic seizures and psychiatric disorders. Other analgesic, in particular opium-derived, also has revealed physical and psychological side effects [113]. Therefore, alternative medicine is still an urgent need for management of DPN. Many natural compounds have been reported in the management of DPN. These compounds include phenolic compounds [114], cannabinoids [115, 116], vanilloids [117, 118] and essential fatty acid [119].

In the diabetic patient, AR is overexpressed in a variety of organs/tissues, particular in peripheral nerve [92], suggesting a possible link between AR and DPN. Sorbitol is the metabolite of glucose converted by AR in polyol pathway. Oka and Kato reported that increased accumulation of sorbitol results in the decrease of *myo*-inositol in the peripheral nerve [120]. Downregulatoin of *myo*-inositol subsequcetly results in lower Na^+, K^+ -ATPase activity, which is important for nerve conduction [120]. Therefore, blocking sorbitol accumulation by inhibiting AR polyol pathway is a strategy being considered for DPN treatment. Genetic studies support this concept, as AR knockout mice appear to be protected from delayed motor nerve conduction velocity [71]. Pharmacological inhibition of AR also showed encouraging or convincing results for clinical use. Epalrestat has been approved for use in the treatment of diabetic neuropathy in Japan [121]. Several clinical trials with epalrestat showed that 150 mg/day improves MNCV and SNCV, and subjective symptoms in patients with DPN [122, 123]. Two additional AR inhibitors, Fidarestat [124–126] and Rainrestat [124, 127, 128], also provide encouraging experimental and/or clinincal results in treatment of DPN and diabetic sensorimotor polyneuropathy (DSP), respectively. With these promising results of AR inhibitors, more about their efficacy and safety will need to be investigated to promote them in the U.S. market. Therefore, the natural products with lower cytotoxicity are potential candidates in AR inhibitor development for DPN treatment.

13.3.3.2 Cardiac Autonomic Neuropathy

Cardiac autonomic neuropathy (CAN) is characterized by dysfunction in the cardiac autonomic nerves causing dysregulation of heart rate. CAN results in higher incidence of heart failure and sudden death in diabetic patients [129]. In diabetic animal models, sorbitol accumulation from polyol pathway has been considered a major contributor to diabetic neuropathy [130]. Since it is considered an effective

AR inhibitor, Sorbinil was used as a therapeutic agent in diabetic patients exhibiting improvement in CAN symptoms [54–56]. Other AR inhibitors such as Epalrestat [131–133], Ponalrestat [134–136] and Tolrestat [137, 138] were also reported as therapeutic agents against CAN. In 1981, Kline et al. developed a useful method entitled I-123 Meta-Iodobenzylguanidin (MIBG) to facilitate imaging the myocardium. This method provides quantitative information of heart rate [139] and is a useful tool in investigating diabetic autonomic disorder in patients [139]. Using MIBG, AR inhibition was observed to alleviate CAN progression in diabetic rats [57] and patients [58]. As a result, AR inhibitors might be a promising treatment for CAN. Thus, development of novel, effective, and nontoxic AR inhibitors is still necessary for slowing the progression of diabetic autonomic neuropathy [140].

13.3.4 Ocular Disorders

13.3.4.1 Uveitis

Uveitis is an ocular inflammatory disease of the uvea, the middle layer of the eye that consists of the iris (anterior uveitis), ciliary body (intermediate uveitis) and choroid (posterior uveitis), that contributes about 10 to 20% legal blindness per year [82]. Therefore, the aim of uveitis treatment is to prevent inflammatory responses. Topical eye drops or oral administration of glucocorticoid steroids is the most common treatment for uveitis [141]. In animal studies, lipopolysaccharide (LPS) is commonly used for induction of experimental uveitis, or so-called endotoxin-induced uveitis (EIU) [39, 51, 52, 142, 143]. LPS injection results in induction of TNF-α [51, 52, 142], ROS [51, 142], cyclooxygenase-2 (COX-2) [51, 52, 143], inducible nitric oxide synthase (iNOS) [51, 52, 143] and NF-κB activation [51, 143] in rodent eyes. Experimental autoimmune uveoretinitis (EAU) is another model for the investigation of ocular inflammatory response [52, 144]. Within these two uveitis models, secretion of proinflammatory cytokines is thought to play an important role resulting in damage to ocular tissue [52, 142]. Regarding the effects of AR on inflammatory responses, many studies using macrophages demonstrated that pharmacological inhibition or genetic ablation of AR attenuates LPS-induced cytokines secretion, oxidative stress and cell migration by suppressing MMP-9 and NF-κB activation [36–40]. In addition, studies also reported that downregulation of AR by enzymatic activity or gene expression is capable of preventing experimental models of EIU or EAU [39, 51, 52]. In the eye, retinal microglia (RMG) are one of the major immune cells that participate in surveillance in retinal environment. While they are typically located in the inner and outer plexiform layers in a healthy condition [145, 146], in uveitis they become activated [147] leading to morphological transformation [142] and migration into the outer nuclear layer (photoreceptor layer) where they secrete cytokines and peroxynitrites [144, 146]. In vitro studies using primary cells showed that RMG can be activated by LPS exposure [40, 148] and such activation can be suppressed by addition of an AR inhibitor or in mice

lacking the functional allele of the AR gene [40]. For these reasons, we propose that AR could be a therapeutic target for uveitis.

13.3.4.2 Diabetic Cataract

In 2010, it was estimated that around 285 million people worldwide had diabetes [149]. There is an estimation that around 552 million people, which is one in ten, will have diabetes by 2030 [150]. Many studies have shown that diabetes is associated with higher prevalence of cataracts, which remains a major cause of blindness in the world [151–153]. Tight glycemic control is known to reduce the risk of cataracts in subjects with type 2 diabetes [154]. Among the factors thought to induce lens opacification, oxidative damage is thought to be a major mechanism in the onset or progression of diabetic cataract (DC) [155]. Thus, researchers observed that the use of dietary antioxidant alleviates the cataract progression [156, 157]. Galactose is another substrate metabolized by AR and results in accumulation of galactitol, which also causes cataract formation. Studies on the anterior part of eyes showed that AR activation plays a key role in DC formation [158, 159]. AR inhibitors prevent cataract formation in streptozotocin (STZ)-diabetic animal models [77, 78] and galactose-fed rats [76]. Other than the effect of sorbitol, fructose metabolized from glucose in AR polyol pathway is another precursor that initiates production of AGE [79], which contributes to cataractous lenses of human subjects with diabetes [74]. Therefore, there is still an urgent need for ARI development. Since AR expression is low in wildtype mice, even in diabetes, Lee and colleagues generated the human AR expressing mice to accelerate cataractogenesis for the sugar cataract study [159]. We recently generated human AR transgenic (AR-Tg) mice that can shorten the time for DC formation by STZ injection [160], thus providing a useful laboratory model for studying DC formation and prevention. The role for AR in DC formation was further substantiated when it was observed that a lower level of diabetic cataract formed in AR null mice compared to wild type [161].

13.3.4.3 Diabetic Retinopathy

Diabetic retinopathy (DR) is one of the major complications of diabetes and has become the leading cause of blindness in people of working age in the past century [75, 162]. Clinical features of DR are macular edema, retinal ischemia, retinal hemorrhages and microaneurysms, formation of intraretinal microvascular abnormalities, growth of neovascular vessels onto the retina, and retinal detachment [163]. Patients with DR experience a decline of visual acuity that affects many activities of daily living. Among the factors causing DR, vascular endothelial growth factor (VEGF) is considered a major one that leads to neovascularization in retina [164, 165]. Animal studies have also shown a correlation between elevated VEGF and diabetic vasculopathy [166]. Clinical trials have shown that use of the VEGF neutralizing antibodies by intravitreal injection improves visual acuity [167, 168].

Although anti-VEGF therapy has revolutionized management of DR, this procedure requires repeated injections, often monthly for 2 years, and may lead to impaired survival of neuronal and vascular cells [169]. Thus, alternative strategies are being sought to offset VEGF-driven pathology in the diabetic retina, such as reduction of VEGF protein production. In animal studies, genetic ablation of VEGF in Muller cells reveals the important role of VEGF production in retinopathy [170, 171]. Genetically predisposed diabetic mice (*db/db*) carrying a mutation in leptin receptor are type 2 diabetic models for investigation of DR [172–174]. Elevation of VEGF in the diabetic retina is decreased when the AR null mutation is introduced into *db/db* mice, which further prevents blood-retinal barrier (BRB) breakdown and apoptosis in retina [175]. Deletion of AR also prevents mice from streptozotocin-induced DR by inhibiting retinal capillary degeneration and superoxide generation [83]. Reduction of AR activity using inhibitors helps to normalize VEGF levels [166], suppressing VEGF-induced tube formation in retinal endothelial cells [84] and alleviating hyperglycemia-induced damage in retinal pigment epithelial cells [80].

Considering the source of VEGF, Muller cells are believed to be the major immune cells that secrete VEGF in the diabetic eye [81]. However, our current unpublished studies show that genetic ablation of AR reduces hypoxia-induced VEGF secretion by attenuating COX-2 expression in retinal microglia (RMG) indicating that RMG could be another source of VEGF in retina.

Other than anti-VEGF therapy, intravitreal injection of steroids is also used for treatment of diabetic macular edema [176]. Steroids are well-known to reduce inflammatory responses by suppressing NF-κB pathway [177, 178]. In the clinic, patients treated with intravitreal injections of steroids such as triamcinolone and dexamethasone showed improvement in diabetic macular edema by reducing central macular thickness [179]. However, these treatments have the side effects of causing cataracts. Nevertheless, suppression of NF-κB pathway is an effective strategy for prevention of onset and/or progression of DR which can be a blinding disease if left untreated.

Systemic inflammation is considered to be an intrinsic response to diabetes [180]. Inflammatory cytokines like interleukin-1β (IL-1β) and TNF-α are increased in the vitreous of patients with DR [181]. Increased TNF-α in retina leads to retinal vascular permeability [182], microglia activation [183] and induction of apoptotic protein markers [183] in retina. Collective data suggested that anti-inflammatory treatment with glucocorticoids [184] or minocycline [183] attenuates severity of retinopathy and helps to restore the BRB. Muller cells and RMG are thought to contribute to inflammatory responses in retina [163]. Our previous study showed that downregulation of AR reduces inflammatory responses in RMG [40] suggesting AR inhibition plays an alternative role in preventing DR by suppressing inflammatory responses in the diabetic eye.

Advanced glycation end-products (AGEs) have been shown to induce VEFG production [81] and matrix metalloproteinases (MMPs) [185] in the diabetic retina. Induction of MMPs alters the BRB by initiating morphological changes in retinal endothelial cells [185], which is often observed in DR. Amadori-glycated protein is the precursor to AGEs [46]. Patients with diabetes have increase of Amadori-glycated

albumin (AGA) in serum [186] which correlates with higher risk of retinopathy [187]. Animal studies also showed the increase of AGA in the diabetic retina [188]. Inflammation in diabetic individuals could be induced by AGEs in kidney via NF-κB pathway [189] or by AGA in retina through Mitogen-activated protein kinases (MAPK) pathway [188]. Previous studies showed that AR inhibition or genetic deficiency suppresses the NF-κB [36–38] and MAPK pathways [39]. Therefore, it would be an interesting question whether AR mediates AGE or AGA-induced inflammatory responses in diabetic retina. Recent studies have shown that RMG were activated in the presence of AGE [190, 191] and AGA [188], and follow the induction of TNF-α. Our current unpublished data observed that AR inhibition suppresses AGA-induced TNF-α secretion and cell migration in RMG suggesting that AR is involved in AGA-mediated DR.

13.3.4.4 Posterior Capsule Opacification

Posterior capsule opacification (PCO), which is a relatively common complication of cataract surgery [192], results from abnormal proliferation and migration of lens epithelia cells (LECs) [193] in the central posterior capsule resulting in degraded visual acuity. LECs undergo differentiation from an epithelial to a myofibroblast phenotype [100] and matrix contraction [194], which further leads to opacification. TGF-β overexpression in Tg mice led to morphological changes in lens that resembled PCO in human [195]. In the process of PCO, TGF-β plays an important role in developing epithelial-to-mesenchymal-transition (EMT) [100], resulting in expression of EMT related markers such as α-smooth muscle actin (α-SMA) [194], and forming cells with a spindled-shaped myofibroblastic morphology [100]. TGF-β also induces MMPs such as MMP-2 and -9, which has been demonstrated that can be induced under mechanical trauma of cataract surgery [194]. Therefore, suppression of EMT and MMPs activation could lead to prevention of PCO. Previous works showed that AR inhibition suppresses LPS or high glucose-induced MMP-9 activation [40, 196, 197]. Kidney studies further reported that TGF-β-induced MMPs and EMT activations were attenuated by AR inhibitor treatment in renal cells [101, 198]. Yadav and colleagues reported a study using pig capsule that AR inhibitor were shown to reduce LECs proliferation and expression EMT markers [199]. SMAD signaling pathway has been identified as playing a critical downstream role in TGF-β-mediated signaling [200]. TGF-β interaction with its receptor leads to SMADS phosphorylation and subsequent translocation to the nucleus to trigger EMT process [201]. Recently, an encouraging study in LECs showed that AR inhibition suppresses TGF-β2-induced SMADs phosphorylation and its downstream regulatory pathways including cell migration, EMT initiation and MMPs activation [202]. A novel function of AR was reported, involving AR interaction with SMADs. This novel model offers a possible mechanism to explain how AR inhibitor treatment suppresses SMADs activation [202]. This finding indicated the AR facilitates TGF-β/SMADs pathway and AR inhibitor disrupts AR-SMADs interaction. Accordingly, AR could be a therapeutic target for PCO.

13.4 Summary and Future Directions

13.4.1 Novel AR Inhibitors

In the view of AR inhibitor and ocular inflammation, we determined whether β-glucogallin (BGG), isolated from Indian gooseberry, is an efficacious AR inhibitor. We observed that BGG is a potential anti-inflammatory agent against endotoxin-induced uveitis in an experimental mouse model. BGG not only alleviated inflammatory responses in macrophages but also suppressed infiltration of immune cells into the eye [39]. However, our extensive research showed the instability of BGG in thermal acidic condition [203]. We observed that the ester linkage in BGG (glycosyl 1-ester) is labile in aqueous solution. Thus, our collaborators Dr. Daniel LaBarbera and his lab designed β-glucogallin amide (BGA), a derivative of BGG produced by replacing the ester with an amide linkage to join the gallic acid and glucose ring. BGA demonstrated similar inhibitory activity *in vitro* and *ex vivo* but much better stability under thermal acidic condition [203]. With compatible activity and greatly improved stability, BGA holds a promise to be an attractive therapeutic lead toward the treatment of ocular inflammation. Further structure-based drug design is presently ongoing to improve the pharmacological profile of BGA, and more sophisticated animal models will be used to test BGA efficacy in vivo.

Our studies of BGG were motivated by strong animal study results which showed that this AR inhibitor can prevent complications of diabetes mellitus. Clinical trials of many AR inhibitors have been unsuccessful in part due to toxicity from their metabolic breakdown products. In our studies of the effect of AR inhibition on inflammation associated with endotoxin-induced uveitis, we included Sorbinil as a positive AR inhibitor control. Sorbinil is no longer considered a candidate for human therapy because previous clinical studies showed that microsomal metabolites of Sorbinil are cytotoxic [204]. Similarly, other AR inhibitors such as Imirestat [205], Tolrestat [206, 207] and Zoporestat [207] also failed in clinical trials due to liver and/or renal toxicity. So far, only Epalrestat has been shown to be safe and efficacious against diabetic peripheral neuropathy and is now marketed in Japan [121, 207]. Therefore, the encouraging results of BGG and preliminary data from BGA provide a new direction for development of AR inhibitors based on a novel pharmacophore structurally unrelated to previously failed AR inhibitors. Since BGG is abundant in many fruits consumed by humans (gooseberry, rhubarb), it is likely that it will not cause liver and renal complications.

In the study of DR, we wanted to know whether BGG is capable of preventing diabetic complications. We developed hyperglycemic condition using high glucose medium on ARPE-19 cells. We found that BGG is an efficacious AR inhibitor reducing hyperglycemia-induced cell death, ROS production, ER stress and mitochondrial dysfunction [80]. Hyperglycemia also elevates the level of advanced glycation end products (AGEs) in the serum of diabetic patients [186]. A study on AGEs has been known to induce ocular inflammation by triggering RMG activation [148]. Our preliminary studies showed that AR inhibition attenuates amadori

glycated albumin (AGA)-induced inflammatory responses in RMG. The mechanism behind this finding remains unknown and should be studied. Since low cytotoxicity of BGG has been shown in cell line model [39], extensive studies of BGG in animal study are feasible. Diabetes shows higher risk in elevating VEGF in retina that causes neovascularization [164, 165]. Our preliminary showed that pharmacological inhibition or genetic ablation of AR prevents hypoxia-induced VEGF secretion from RMG. Based on our previous study of ARI on RMG, we believe that BGG and BGA are a potential therapeutics for diabetic complication by preventing RMG activation in diabetic retina. However, to further apply BGA to next step, more pharmaceutical kinetics and toxicity experiments are necessary. Although BGA is derived from natural compound, the toxicity *in vivo* has not been studied. Animal toxicity study of BGA injection should be conducted before BGA's further application. Since BGA was designed for its higher stability, *in vivo* pharmaceutical kinetics should also be studied in the future.

13.4.2 A role for AR in Ocular Inflammation

In the eye, RMG is one of the immune cells that normally reside in the inner retina. However, under inflammatory conditions, RMG can be found in higher numbers in the subretinal space between photoreceptor outer segments and the RPE. Activated RMG secrete chemokines and/or cytokines to damage neural and retinal cells in the eye. Therefore, regulation of RMG may be critical for preventing ocular inflammation. To investigate a more specific area of the eye, we conducted an *ex vivo* study to examine a potential role for AR in the response of RMG to endotoxin exposure. We observed that inflammatory responses in RMG following exposure to endotoxin were substantially suppressed when cells were treated with AR inhibitors or were genetically deficient for AR gene expression. These results demonstrated that pharmacological inhibition or genetic ablation of AR prevents endotoxin-induced inflammation in the retina by suppressing RMG activation [40]. An MMP-9 inhibitor, designed to inhibit gelatinase activity, was shown to prevent LPS-induced cell migration. By a different route, AR inhibition prevents cell migration by reducing MMP-9 protein expression. Therefore, one would expect additive effects of combined treatment with AR and MMP inhibitors on preventing cell migration. A preliminary animal study was extensively performed using CX3CR1 transgenic mice, a strain in which monocytes, including RMG, constitutively express green fluorescence protein and therefore are easily observed in ocular tissue sections. We observed that LPS injection triggers RMG activation and migration into inner and outer nuclear layers (Fig. 13.4). Co-injection with Sorbinil alleviated LPS-induced RMG activation and migration in the retina (Fig. 13.4) indicating that AR inhibition is valid *in vivo* to prevent ocular inflammation. In inflammation, TNF-α plays a robust role in causing apoptosis. We have previously shown that downregulation of AR by either pharmacological inhibition or genetic ablation reduces TNF-α secretion in RMG as well as apoptosis in co-cultured RPE cells [40]. Studies on the

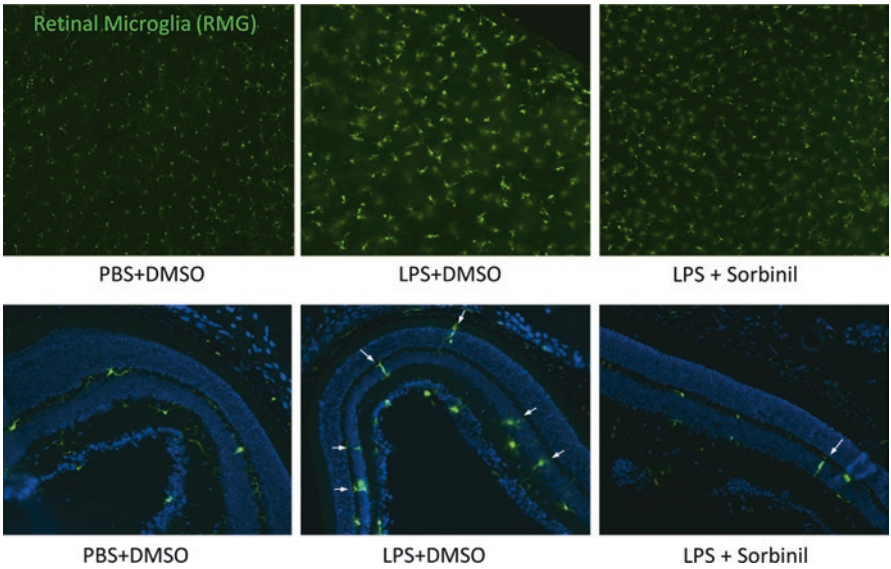

Fig. 13.4 AR inhibition prevents RMG activation and migration under LPS exposure. Cx3Cr1 transgenic mice were injected with LPS (500 ug / mouse) for 24 h with or without Sorbinil co-injection. Green spots indicate RMG in retinas. White arrows indicate RMG migration into inner or outer nuclear layers. Figure adapted by Chang and Petrash from *Biochem Biophys Res Commun*. 2016; 473(2):565–571

detection the level of TNF-α secretion and apoptosis in retina should be conducted in the future. Since we have AR null mice, genetic effects of AR on LPS-induced RMG activation would be a convincing experiment to confirm the role of AR in retinal inflammation. The study of RMG provides a therapeutic target in the retina for AR-associated ocular inflammatory diseases such as uveitis and retinopathy. In addition, Muller cells are the glial cells that have immune functions in the eye. AR was also reported to express in the Muller cells [208, 209]. Several studies showed that Muller cells are involved in uveitis, proliferative vitreoretinopathy (PVR) and DR [210–212]. Therefore, the effect of AR on Muller cells is an interesting study to be conducted in the future.

In the experimental uveitis model or hyperglycemic stress experiment, we treated the mice or cell line at the same time while uveitis or hyperglycemic stresses were being induced. However, in real practice, treatment is always applied after disease occurs. Based on this point, the efficacy of AR inhibitors after stressor such as LPS or hyperglycemia needs to be tested. Although we haven't conducted experiments regarding this issue, administration of AR inhibitors after retinal inflammation or hyperglycemia would better mimic the treatment paradigm in uveitis or diabetes management. It is possible that the effect of post-treatment would be less effective than pre-treatment due to some possible irreversible changes that may have occurred or the disease may have gone to a more advanced stage that is less susceptible to ARI therapy.

We observed that AR inhibitors lowered the abundance of inflammatory cells in the vitreous of endotoxin-treated animals. One possibility is that inhibitors reduce expression of MMPs and thereby reduce the ability of cells to migrate toward their targets. Another possible explanation for reduced immune cell infiltration may relate to their ability to pass through the vascular endothelium. Adhesion molecules are cell surface receptors that facilitate the binding of immune cells to endothelial cells and penetration [213]. These adhesion molecules include intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule (VCAM). TNF-α is an inflammatory cytokine that mediates pathological endothelial changes which cause induction of adhesion molecule expression and vascular leakage at the site of inflammation [214]. Ramana and colleagues reported that AR inhibition prevents TNF-α-induced increases of ICAM-1 and VCAM in human umbilical vein endothelial cells (HUVECs) as well as decreases monocytes adhesion to these cells [215]. This observation may provide another explanation for the effect of AR inhibitors on prevention of inflammatory cells infiltration into the eye.

13.4.3 Studies of AR as a Mediator of EMT During Development of Posterior Capsule Opacification

Regarding the effect of AR inhibitor on PCO development, we proposed a novel idea of AR with a noncatalytic function. We observed AR interacts with Smads in a NADPH-dependent pathway but in a manner not requiring enzymatic activity [202]. This is the first paper that reports a non-enzymatic function of AR. We also found that AR inhibition or genetic ablation prevents TGF-β2-induced Smads activation and expression of EMT markers, thus indicating a potential therapeutic strategy for prevention of PCO development. However, remaining unknown are the details of how AR interacts with Smads and whether there is a distinct interaction site. Sequence deletion could be used for understanding the possible site or domain. Since AR null mice are available, we can develop PCO in either wildtype or AR null mice to confirm the hypothesis *in vivo*. Sorbinil disruption of AR-Smads interaction could be resulted from an AR conformational change or actual blocking of a protein-protein interaction site. In this study, we only tested the noncatalytic function of AR using Sorbinil. However, whether other ARIs such as BGG also contribute similar effect is still unknown. Other ARIs may fail to disrupt AR-Smads interaction due to different results of conformational change or blocking site. Therefore, more studies on different ARIs on noncatalytic effect need to be conducted to elucidate the hypothesis.

Most of the published studies surrounding Sorbinil as an anti-inflammatory agent implicitly presume its efficacy derives from inhibition of AR catalytic activity. In our PCO study, we showed that a catalytically inactive mutant of AR was able to facilitate TGF-β2-induced Smad activation, demonstrating that the ability of Sorbinil to downregulate Smad activation was unrelated to its ability to inhibit AR

catalysis. Therefore, the effect of Sorbinil can be segregated into its effects on catalytic and noncatalytic functions of AR. Previous studies reported that AR plays a catalytic role in reduction of aldehydes during lipid peroxidation pathway following NF-κB activation [20, 21] and AR inhibitors such as Sorbinil suppress this pathway [23, 25–27]. However, the noncatalytic role of AR in NF-κB activation has not been studied to date. Going forward, it would be possible to address this question by transfecting AR null cells with wildtype AR (wtAR) or an active site mutant AR (mutAR) and treat the cells with or without AR inhibitors (Sorbinil or BGG) under LPS exposure. If LPS induces the same level of NF-κB activation in mutAR group compared to wtAR group, one could conclude that AR facilitates NF-κB activation in a noncatalytic fashion. Similarly, if Sorbinil treatment prevented NF-κB activation in both groups, it would be consistent with the notion that an AR interaction domain overlapping the ARI binding site is important for NF-κB activation in a manner similar to our findings with AR and Smad activation.

AR has been studied extensively as a catalyst that results in sorbitol accumulation and contributes to pathogenesis of diabetic complications. Our current pharmacological studies confirm the importance of AR through its role as a regulator of glucose metabolism through the polyol pathway (catalytic function). In addition, we have compelling results which point to AR as a component of the TGF-β signaling pathway (noncatalytic function). It will be exciting to see the results of future studies which aim to further clarify the mechanism linking AR to TGF-β signaling and possible therapeutic strategies to prevent TGF-β-associated disease.

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Chapter 14
Engineered Animal Models Designed for Investigating Ethanol Metabolism, Toxicity and Cancer

Stephanie Marshall, Ying Chen, Surendra Singh, Pablo Berrios-Carcamo, Claire Heit, Nicholas Apostolopoulos, Jaya Prakash Golla, David C. Thompson, and Vasilis Vasiliou

Abstract Excessive consumption of alcohol is a leading cause of lifestyle-induced morbidity and mortality worldwide. Although long-term alcohol abuse has been shown to be detrimental to the liver, brain and many other organs, our understanding of the exact molecular mechanisms by which this occurs is still limited. In tissues, ethanol is metabolized to acetaldehyde (mainly by alcohol dehydrogenase and cytochrome p450 2E1) and subsequently to acetic acid by aldehyde dehydrogenases. Intracellular generation of free radicals and depletion of the antioxidant glutathione (GSH) are believed to be key steps involved in the cellular pathogenic events caused by ethanol. With continued excessive alcohol consumption, further tissue damage can result from the production of cellular protein and DNA adducts caused by accumulating ethanol-derived aldehydes. Much of our understanding about the pathophysiological consequences of ethanol metabolism comes from genetically-engineered mouse models of ethanol-induced tissue injury. In this review, we pro-

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vide an update on the current understanding of important mouse models in which ethanol-metabolizing and GSH-synthesizing enzymes have been manipulated to investigate alcohol-induced disease.

Keywords Transgenic · Alcohol · Aldehyde dehydrogenase · Glutathioine · Cancer · Tumor · Acetaldehyde

14.1 Introduction

Alcoholic beverages have long been an integral part of our society, and serve many functions in medicine and social interactions. Not surprisingly, the effect of alcohol on human health and society has been subject to intensive research. Alcohol consumed in moderation (7 glasses/wk) is considered to have only a minor impact on health [1]. There is even some evidence that ethanol consumption (depending on the nature and speed at which it was consumed) may have health benefits [1]. However, excessive consumption of alcohol (or alcohol abuse) can lead to a plethora of adverse health outcomes, including injuries, violence, suicide, poisoning, cirrhosis, cancer, and possibly hemorrhagic stroke [2, 3]. Because of this, levels of alcohol consumption have been established by the NIAAA that represent abuse, i.e., typically ≥5 alcoholic drinks/day for males and ≥ 4 alcoholic drinks per day for females [4]. Alcohol abuse-related deaths total ≈ 3.3 million per year worldwide (equaling nearly 6% of total deaths), making it the fourth leading cause of preventable death [5]. Alcohol places a significant financial burden on the economy; its estimated healthcare costs surpassed $223 billion in 2006 in the United States alone [1]. The development of interventions that prevent or treat diseases associated with alcohol consumption is dependent upon our understanding of the mechanisms by which ethanol induces pathophysiological changes. Consumed alcohol (ethanol) is metabolized to acetaldehyde, primarily by alcohol dehydrogenase (ADH) and cytochrome p450 2E1 (CYP2E1). Aldehyde dehydrogenases (ALDHs) eliminate acetaldehyde by oxidizing it to acetate (Fig. 14.1). Acetaldehyde is toxic in large quantities, and clinically causes a flushing syndrome manifesting as facial flushing, nausea, and tachycardia [6]. At the cellular level, it leads to increases in reactive oxygen species (ROS), depletion of antioxidants (e.g., glutathione), and formation of DNA and protein adducts. Acetaldehyde is classified as an Interntional Agency for Research on Cancer (IARC) group I carcinogen [7] and has been shown to promote cancer [8]. In addition, byproducts of ethanol metabolism are hazardous to human health. For example, the accumulation of ROS, DNA adducts and lipid peroxidation products can cause significant tissue damage by interfering with normal tissue functions. Genetically-modified mouse models have served as important tools for enhancing our understanding of the mechanisms by which alcohol induces tissue injury. While significant progress has been made, we still have much to discover regarding how specific mechanisms, such as stress, inflammation and cell signaling pathways interact and lead to alcohol-related pathologies, such as alcoholic liver disease, pancreatitis, cardiovascular disease, and diabetes mellitus, as well as various cancers,

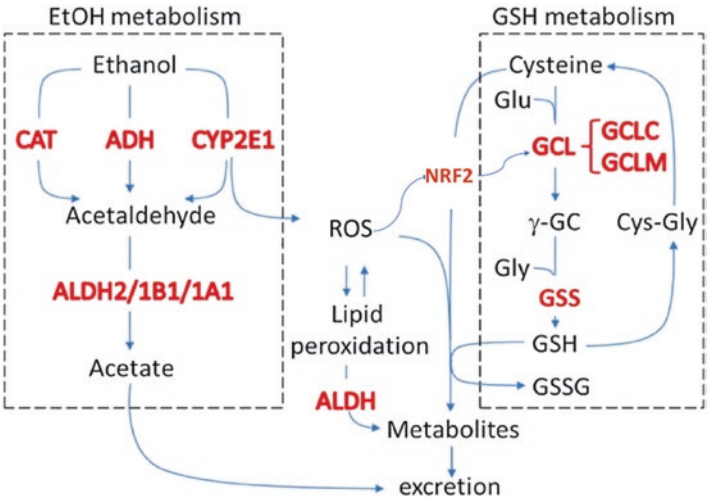

Fig. 14.1 Description of enzymes that participate in ethanol metabolism. Ethanol is metabolized to acetaldehyde by catalase (CAT), alcohol dehydrogenase (ADH) and/or cytochrome P450 2E1 (CYP2E1). A lack of any of these enzymes will diminish the rate of synthesis of acetaldehyde and delay the elimination of ethanol. Acetaldehyde is primarily metabolized to acetic acid by aldehyde dehydrogenase 2 (ALDH2), although ALDH1B1 and ALDH1A1 may also contribute. Reductions in the activity of these enzymes result in elevated levels of acetaldehyde after ethanol consumption. ALDH1B1 and ALDH1A1 also participate in the metabolism of retinaldehyde to retinoic acid. Reactive oxygen species (ROS), by-products of ethanol metabolism, are scavenged by glutathione (GSH)-dependent mechanisms. Enzymes involved in GSH synthesis, such as glutamate-cysteine ligase catalytic (GCLC) and modifier (GCLM) subunits are integral to neutralizing and maintaining homeostatic ROS levels that are generated after ethanol consumption preventing the deleterious effects of ethanol (adapted from [17])

including oral, colorectal, liver, pancreatic, aerodigestive, breast, and colon [2–7]. We present here an update on the mouse models that hold the potential to lead to a deeper understanding of the pathways involved in the metabolism of ethanol and of the mechanisms that make cells vulnerable to alcohol-induced damage.

14.2 Animal Models for Alcohol-Induced Cancer

The pathological consequences of alcohol abuse occur over a spectrum. Aside from a few pathologies, such as acute steatohepatitis, prolonged alcohol use is usually associated with chronic disease. Specific lifestyle and genetic factors can increase the likelihood that alcohol consumption leads to disease [9]. Much research has been done on ethanol's contribution to esophageal, head and neck and oral cancers [10–12]. Heavy drinkers (i.e., those who have consumed 3 ethanol-containing beverages/day for women and 7 ethanol-containing beverages/day for men, for more

for ≥5 days in the past month) appear to be at an increased risk for these cancers; they retain this increased risk even after 10 years of abstinence [13]. While a wealth of epidemiological data exists that relate alcohol consumption to cancer risk, a lack of appropriate experimental models has hindered our progress in understanding the molecular mechanisms by which ethanol promotes cancer formation [14]. Nevertheless, strong evidence suggests that acetaldehyde and free radical production from ethanol metabolism are significant contributors to alcohol-associated tumorigenesis [8]. The genetically-modified mouse models of ethanol, acetaldehyde and glutathione metabolism (presented herein, Table 14.1) hold the potential to facilitate the elucidation of the mechanisms by which ethanol promotes cancer development.

14.3 Mouse Models with Genetic Deficiencies in Ethanol-Metabolizing Enzymes

Ethanol is first oxidized to acetaldehyde through the enzymatic action of multiple isozymes of cytosolic alcohol dehydrogenase (ADH). This occurs primarily in the liver. Catalase and cytochrome p450 2E1 (CYP2E1) can also metabolize ethanol to acetaldehyde, albeit when ethanol is present in high concentrations, or in cells that only poorly express ADH [15]. The products of these reactions (e.g., acealdehyde, ROS) are rapidly metabolized to less harmful products. Aldehyde dehydrogenase (ALDH) converts acetaldehyde to acetate (which enters into the citric acid cycle). Antioxidants, such as glutathione, are produced to detoxify the ROS generated from CYP2E1.

14.3.1 Adh1 *Global Knockout*

As noted, alcohol dehydrogenase (ADH) is primarily responsible for the metabolism of ethanol to acetaldehyde. ADH1 catalyzes unbranched alcohols to their corresponding aldehydes. Duester and colleagues generated the *Adh1*$^{-/-}$ mouse line [16]. Through evolution, humans have gained three genes for ADH1*, viz. ADH1A, ADH1B,* and *ADH1,* while mice retain just one ADH1 gene [11]. *Adh1*$^{-/-}$ mice are phenotypically normal and fertile but have a limited capacity to oxidize ethanol and retinol. They have been reviewed in [17]. Upon ethanol feeding, *Adh1*$^{-/-}$ mice exhibit reduced blood ethanol clearance, demonstrating that ADH1 has ethanol dehydrogenase activity [18]. These mice have been used successfully in mouse model studies of vitamin deficiencies and ethanol-induced tissue damage studies to elucidate the mechanism of action of retinoid dehydrogenases and aldehyde dehydrogenases.

Table 14.1 Overview of the phenotypes of the differing genetic strains of mice in the Vasiliou Lab

Strain	Genetic background	Phenotype	Reference
Catalase global knockout	C57BL6	Do not express catalase.	Ho 2004, Heit 2017
		Develop normally, i.e., exhibit no gross abnormalities. Brain mitochondria show deficiencies in respiration.	
		Have age-related weight gain when fed regular chow.	
Cyp2e1 global knockout	C57BL6	Do not express CYP2E1.	Rindler 2016, Abdelmegeed 2013, Lu 2015
		Viable and develop normally.	
		Exhibit a lower sensitivity to the deleterious hepatic effects of acetaminophen.	
		Are protected against apoptosis and steatohepatitis and autophagy in a chronic ethanol-fed model.	
Cat and Cyp2e1 global double-knockout	C57BL6/129 mixed	Do not express catalase or CYP2E1.	Unpublished
		Are viable, fertile and show no gross abnormalities. We are currently investigating alcohol metabolism, ethanol-induced sleep times and preference in these mice.	
Aldh2 global knockout	C57BL6	Do not express ALDH2.	Isse 2005, Kiyoshi 2009, Jamal 2016, Matsumoto 2014, Oyama 2007
		Are viable and develop normally.	
		Exhibit lower ethanol and acetaldehyde clearance than wild-type mice.	
		Ethanol administration causes dose-dependent reduction in lifespan, body weight, and increased serum ALT activity.	
		More sensitive to the toxic effects of inhaled acetaldehyde.	
Aldh1b1 global knockout	C57BL6J	Do not express ALDH1B1.	Singh 2015, Anastasiou 2016
		Are viable and develop normally.	
		Exhibit lower acetaldehyde clearance.	
		Show glucose intolerance and hyperglycemia.	
Aldh1a1 global knockout	C57BL6	Do not express ALDH1A1.	Fan 2003
		Are viable and develop normally.	
		Following retinol treatment, livers display reduced retinoic acid synthesis and increased serum retinal levels.	
Aldh3a1 global knockout	C57BL6	Do not express ALDH3A1.	Koppaka 2016, Chen 2015
		Are viable and develop normally.	
		Develop cataracts or corneal hazing	

(continued)

Table 14.1 (continued)

Strain	Genetic background	Phenotype	Reference
Aldh1a1 and *Aldh3a1* double knockout	C57BL6	Do not express ALDH1A1 or ALDH3A1.	Koppaka 2016
		Are viable and develop normally.	
		Develop cataracts	
GCLC conditional *(Gclc$^{f/f}$)* knockout	C57BL6	Do not express GCLC in cre-targeted tissues.	Chen 2010, Chen 2014
		Mice are produced at expected mendillian ratios.	
		Gclc$^{h/h}$ mice die from liver failure due to lack of proper mitochondrial function	
		Gclc$^{le/le}$ mice have defects in ocular development	
GCLM global *(Gclm$^{-/-}$)* knockout	C57BL6	Do not express GCLM.	Yang 2002
		Viable, fertile and develop normally.	
		Protected against liver injuries induced by a variety of hepatic insults	
Gclm and *Nrf2* knockout mice	C57BL6	Do not express GCLM or NRF2.	Unpublished
		Are viable and reach maturity; however, have yet to produce progeny.	
		May develop hepatitis by 6 months of age.	
Mice still under investigation		*Apc$^{F/F}$Cdx2$^{ERT2\text{-}Cre}$/Aldh1b1$^{-/-}$* mutant line, *Aldh1b1 and Aldh1a1* double knockout, *Aldh1b1* and *Aldh2* double knockout, *Aldh3a1* C244A knock-in, *Aldh1a1C303A* knock-in mouse strain	

14.3.2 Catalase *Global Knockout*

A small proportion of alcohol consumed is metabolized by catalase. The main biological role of catalase is the conversion of hydrogen peroxide (an important, harmful ROS metabolite) to water and oxygen. The catalase knockout (*Cat$^{-/-}$*) mouse strain was developed and characterized by Ho and colleagues [19]. These mice do not express catalase and develop normally, i.e., exhibit no gross abnormalities [17]. However, brain mitochondria of these animals show deficiencies in respiration. In spite of the absence of catalase, these animals do not show increased vulnerability to hyperoxia-induced lung injury [19]. Nevertheless, catalase appears to modulate ethanol sensitivity in the brain [20, 21]. Administration of 10% ethanol in drinking water to *Cat$^{-/-}$* mice (considered binge ethanol administration) increases serum alanine aminotransferase activity, plasma homocysteine levels, indicating liver damage, hepatic fat accumulation, and lipid peroxidation. Catalase has a role in protecting the liver against binge ethanol exposure [22]. Through the use of an

anti-catalase shRNA gene-coding lentiviral vector, acetaldehyde was shown to be important in influencing preference for ethanol in rats [23]. Recently, it was revealed that in *Cat*$^{-/-}$ mice fed a high-fat diet, mitochondrial-derived H_2O_2 is responsible for diminished insulin signaling in the heart [24]. Rindler and colleagues identified catalase as an important component of the immediate antioxidant response [25]. Thus, the *Cat*$^{-/-}$ mice (alone or in combination with the other knockouts) are a valuable tool for examining determinants of ethanol drinking preference as well as ethanol-induced tissue damage.

14.3.3 Cyp2e1 *Global Knockout*

Cytochrome p450 2E1 (CYP2E1) is an ethanol-inducible enzyme with a role in hepatic ethanol oxidation. By genetically ablating exon 2 of the *Cyp2e1* gene, Gonzalez and colleagues developed *Cyp2e1*$^{-/-}$ mice [26]. These mice do not express the CYP2E1 enzyme but develop normally [26]. Interestingly, they show a reduced sensitivity to the deleterious hepatic effects of the analgesic acetaminophen [26]. As one of the primary xenobiotic/endobiotic-metabolizing cytochrome P450 enzymes, CYP2E1 protects against a variety of endogenous or exogenous pathogens. Using the *Cyp2e1*$^{-/-}$ mouse model, CYP2E1 has been shown to play a pivotal role in mediating ethanol-induced hepatotoxicity [27, 28]. *Cyp2e1*$^{-/-}$ and *Cyp2e1* knock-in mice have been used to examine the potentiation of ethanol-induced hypoxia. *Cyp2e1* knock-in mice exhibited the lowest levels of hypoxia and HIF1-α protein expression compared to both WT mice and *Cyp2e1*$^{-/-}$ mice [29]. In humans, up to 60% of ethanol metabolism can be attributed to CYP2E1 in excessive alcohol consumers; it has a higher K_m for alcohol than ADH and its activity is induced by chronic alcohol ingestion [30–32]. It has been reported that the regulatory sequences in the promoter of CYP2E1 may play a role in the pre-absorptive metabolism of alcohol [33]. *Cyp2e1*$^{-/-}$ mice are protected against apoptosis and steatohepatitis [34], and autophagy [35] in a chronic ethanol-fed mouse model. Similarly, ethanol-induced fatty liver and oxidant stress are blunted in these mice [36]; this study confirmed the important role CYP2E1 plays in ethanol-induced liver toxicities. *Cyp2e1*$^{-/-}$ mice also display longer ethanol-induced sleep time [15], reinforcing the relevance of the *Cyp2e1*$^{-/-}$ mouse line for the study of the CYP2E1 enzyme in ethanol toxicities and alcohol-related drinking preference.

14.3.4 Cat *and* Cyp2e1 *Global Double-Knockout*

We have recently generated the *Cat*$^{-/-}$ and *Cyp2e1*$^{-/-}$ double-knockout mouse line by crossing the two single knockout mouse lines (Matsumoto A, Chen Y, Vasiliou V et al., manuscript in preparation). No expression of catalase and CYP2E1 proteins were detectable in their liver or brain. These mice are viable, fertile and show no

gross abnormalities. We are currently investigating alcohol metabolism, ethanol-induced sleep times and ethanol preference in these mice.

14.4 Mouse Models with Genetic Deficiencies in Acetaldehyde-Metabolizing Enzymes

After the first step of ethanol metabolism to acetaldehyde by ADH, aldehyde dehydrogenases (ALDHs) metabolize this toxic intermediate to a less active byproduct, acetate. This is normally a rapid step; however, under conditions of excessive ethanol consumption, acetaldehyde may accumulate, leading to increased ROS, lipid peroxidation, DNA adducts and physical manifestations, such as flushing and tachycardia. Multiple ALDH isozymes metabolize ethanol-derived acetaldehyde. The majority of acetaldehyde generated from ethanol is metabolized by ALDH2 in the mitochondria. Cytosolic ALDH1A1 also metabolizes acetaldehyde, but to a lesser extent than ALDH2. The ALDH1B1 isozyme shares 72% amino acid sequence homology with ALDH2 and has been shown to have an affinity for both acetaldehyde and retinaldehyde [1].

14.4.1 Aldh2 *Global Knockout*

The *Aldh2*$^{-/-}$ strain was first developed and characterized by Isse et al. [37, 38]. These mice are fertile and display no overt phenotype. Following oral administration of ethanol, *Aldh2*$^{-/-}$ mice exhibit higher ethanol and acetaldehyde levels and lower acetate levels in the blood, brain, and liver [39–41], and show a dose-dependent reduction in lifespan, body weight, and increased serum ALT activity [42]. *Aldh2*$^{-/-}$ mice are also more sensitive to the toxic effects of inhaled acetaldehyde [43] and to the genotoxic effects of exposure to the biofuel ethyl *tert*-butyl ether (which generates acetaldehyde in the body) [44]. In addition, beneficial effects of low to moderate ethanol consumption, such as increased HDL-cholesterol levels and cardioprotection, are not observed in *Aldh2*$^{-/-}$ mice [45, 46]. *Aldh2*$^{-/-}$ mice show altered liver function under basal conditions [47] and after ethanol exposure [48]. Conversely, higher levels of hepatic antioxidant proteins have been found in naive *Aldh2*$^{-/-}$ mice fed standard chow [49]. Bone structure alterations have also been observed in naïve *Aldh2*$^{-/-}$ mice [50] and in those treated with alcohol [51]. Cardiac function of *Aldh2*$^{-/-}$ mice is more adversely affected by exposure to an endoplasmic reticulum stress inducer [52] or alcohol [53]. On the other hand, *Aldh2*$^{-/-}$ mice show diminished compensatory cardiac hypertrophy in an aortic ligation model [54]. Hence, there is no consensus on a positive or negative effect of ALDH2 absence on cardiac function. A diabetes phenotype induced in *Aldh2*$^{-/-}$ mice exhibit more severe energy metabolism impairments and diastolic dysfunction

[55]. *Aldh2*$^{-/-}$ mice show higher acetaldehyde-DNA adducts in the esophagus [56, 57], stomach [58, 59] and liver [60, 61] after ethanol administration. In addition, mice with a polymorphism in ALDH2 (i.e., ALDH2*2) are more prone to liver tumor formation [62]. This suggests that acetaldehyde generation is likely involved in the pathogenesis of upper gastrointestinal, gastric and liver cancer. All of these findings demonstrate that the value of *Aldh2*$^{-/-}$ mice as a strain that can be used to identify the contribution of ALDH2 to ethanol metabolism and toxicity.

14.4.2 Aldh1b1 *Global Knockout*

Our laboratory generated the *Aldh1b1*$^{-/-}$ strain [63]. These mice develop and breed normally without any overt phenotype. In agreement with the alcohol hypersensitivity and aversion associated with the ALDH1B1 polymorphism in Caucasians and with the catalytic properties of ALDH1B1 (i.e., the second lowest K_m for acetaldehyde oxidation) [64–66], *Aldh1b1*$^{-/-}$ mice exhibit slower acetaldehyde clearance. Microarray analysis of a murine whole embryo culture model revealed ALDH1B1 to be expressed only in the ethanol-treated embryos with open neural tubes [67]. These findings suggest a crucial role of ALDH1B1 in ethanol toxicity during embryonic development. The *Aldh1b1*$^{-/-}$ mouse strain represents the first animal model that allows the study of ALDH1B1 in ethanol-induced tissue injury. We found that ALDH1B1 may promote tumor formation by downregulating the Wnt/β-catenin, Notch and PI3K/Akt signaling pathways [63]. ALDH1B1 is expressed in embryonic and adult pancreatic progenitor murine cells and has been proposed as a marker for these cells. Anastasiou and colleagues [68] showed alterations in beta cell functionality in the pancreas of *Aldh1b1*$^{-/-}$ mice with decreased glucose sensing, stimulus-secretion coupling and secretory granule biogenesis, as well as decreased expression of *Nrf2* and other genes related to oxidative stress protection. Such changes may underlie the glucose intolerance and hyperglycemia that manifests in *Aldh1b1*$^{-/-}$ mice over the long term [69, 70]. Centroacinar-like cells that express ALDH1B1 are present in the adult mouse pancreas, and their number increases dramatically following experimental pancreatic injury, such as that caused by caerulein or streptozotocin treatment [69]. These cells are also believed to be involved in pancreatic cancer initiation [69]. Recently, we showed that ALDH1B1 is strongly expressed in human pancreatic adenocarcinoma cells and is crucial for their proliferation [71]. As such, ALDH1B1 may play a central role in pancreatic development, regeneration and cancer [69–71]. The *Aldh1b1*$^{-/-}$ mouse model provides an opportunity to examine the link between alcohol consumption and diabetes.

14.4.3 Apc$^{F/F}$Cdx2$^{ERT2–Cre}$/Aldh1b1$^{-/-}$ *Mutant*

ALDH1B1 expression is confined to the base of crypts in the normal colon near (or in) the stem cells. In addition, it is highly expressed in human colon adenocarcinomas [72]. Our laboratory has shown that shRNA-mediated suppression of ALDH1B1 in a human colon cancer cell line (SW480) resulted in a decreased number and size of spheroids in 3D-matrigel culture, and in reduced sizes of xenograft tumors in nude mice [63]. We are currently breeding the *Aldh1b1*$^{-/-}$ and the *Apc*$^{F/F}$-*Cdx2*$^{ERT2\text{-}Cre}$ mouse lines to generate an *Apc*$^{F/F}$*Cdx2*$^{ERT2\text{-}cre}$*/Aldh1b1*$^{-/-}$ mouse line. *Apc*$^{F/F}$*Cdx2*$^{ERT2\text{-}cre}$ mice carry a CDX2P-NLS Cre recombinase and a loxP-targeted Apc allele. These mice develop tumors predominantly in the large intestine (especially in distal colon and rectum). Tumors in *Apc*$^{F/F}$*Cdx2*$^{ERT2\text{-}cre}$ mice resemble human colorectal cancers in distribution, molecular changes and gender predisposition, i.e., are more common in males than in females [73]. Due to the finding that ALDH1B1 may be involved in tumor formation, crossing *Aldh1b1*$^{-/-}$ mice with *Apc*$^{F/F}$*Cdx2*$^{ERT2\text{-}cre}$ mice will allow us to more quickly study the role of ALDH1B1 in the process of tumor formation induced by ethanol feeding. Accordingly, *Apc*$^{F/F}$*Cdx2*$^{ERT2\text{-}Cre}$*/Aldh1b1*$^{-/-}$ mutant mice will make an ideal model to study alcohol-induced colorectal cancer.

14.4.4 Aldh1a1 *Global Knockout*

The *Aldh1a1*$^{-/-}$ strain was developed and originally characterized by Fan and coworkers [74]. *Aldh1a1*$^{-/-}$ mice are viable and develop normally. Following retinol treatment, livers from these mice display reduced retinoic acid synthesis and increased serum retinal levels [74], supporting a key role for hepatic *Aldh1a1* in oxidizing retinaldehyde. Interestingly, *Aldh1a1*$^{-/-}$ mice are protected against diet-induced obesity and its comorbidity, and showed insulin resistance, suggesting that ALDH1A1 may regulate the metabolic response to a high-fat diet [75]. ALDH1A1 is highly expressed in the cornea, and its protective role in the eye is illustrated by the observation that *Aldh1a1*$^{-/-}$ mice develop cataracts [76]. Gene expression profiling has shown *Aldh1a1* to be highly expressed in mouse hematopoietic stem cells [77, 78]. Interestingly, hematopoietic stem cells from *Aldh1a1*$^{-/-}$ mice exhibit increased sensitivity to cyclophosphamide (a chemotherapy agent) and its decreased metabolism in the liver [79]. Based upon these results, it has been suggested that ALDH1A1 may be an important regulator of stem cell function. ALDH1A1 is also associated with metabolism of catecholamines and is expressed in the neurons of the mesencephalon where it converts potentially toxic 3,4-dihydroxyphenylacetaldehyde (DOPAL) metabolites to a non-toxic metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC). Hence, by altering the metabolism of catecholamines, loss of ALDH1A1 expression can potentially increase neurotoxicity [80]. In humans, genetic variants of ALDH1A1 that exhibit low enzyme

activity have been associated with increased alcohol sensitivity [81]. In addition, ALDH1 promoter polymorphisms have been proposed to contribute to the observed protection against alcohol disorders in Southwest California Indians [82]. In the Finnish population, alcohol consumption behavior and alcohol dependence risk are influenced by genetic variations in ALDH1A1 [83]. Therefore, the *Aldh1a1*$^{-/-}$ mouse line represents a useful animal model for investigation of the ALDH1A1 enzyme in ethanol and retinol toxicities.

14.4.5 Aldh1b1 *and* Aldh1a1 *Double Knockout*

ALDH1A1 is highly expressed in some commonly-occurring carcinomas and is a marker for poor prognosis in humans (see Tomita et al. for review [84]). ALDH1B1 is expressed in several carcinomas that also express ALDH1A1; in some of these, the level of ALDH1B1 is higher than ALDH1A1, especially in colon adenocarcinoma [72]. An *Aldh1b1* and *Aldh1a1* double knockout (*Aldh1b1*$^{-/-}$*/Aldh1a1*$^{-/-}$) is being developed to identify the role of retinaldehyde- *and* acetaldehyde- metabolizing enzymes in carcinogenesis. This strain will be the product of the crossbreeding of *Aldh1b1*$^{-/-}$ and *Aldh1a1*$^{-/-}$ mice.

14.4.6 Aldh1b1 *and* Aldh2 *Global Double-Knockout*

Beyond the pancreatic impairment that manifests over the long term, the normality of the phenotype of *Aldh1b1*$^{-/-}$ mice could be attributable to compensatory changes in the activity of redundant enzymes, such as ALDH2. The *Aldh1b1* and *Aldh2* double knockout strain (*Aldh1b1*$^{-/-}$*/Aldh2*$^{-/-}$) is being developed to allow examination of the physiological and pathophysiological consequences of complete removal of mitochondrial acetaldehyde oxidation activity. This animal model will be the product of the breeding of *Aldh2*$^{-/-}$ and *Aldh1b1*$^{-/-}$ mice.

14.4.7 *Global* Aldh3a1 *Single and* Aldh1a1/Aldh3a1 *Double Knockouts*

Aldh3a1 is a member of the mouse aromatic hydrocarbon receptor (AHR) gene battery, which encode a highly regulated and coordinated group of drug-metabolizing enzymes [85]. The highest expression of *Aldh3a1* occurs in the cornea, stomach, colon, urinary bladder, and skin of the mouse [86]. Numerous studies suggest a role for ALDH3A1 in a variety of homeostatic mechanisms, including cell proliferation, differentiation and apoptosis, and in resistance to chemotherapeutics used to treat

human breast, hepatocellular or prostate adenocarcinoma [87–89]. ALDH3A1 appears to mediate these effects through both enzymatic and non-enzymatic properties. Enzymatically, ALDH3A1 is involved in the detoxification of lipid peroxidation by-products and aromatic aldehydes [90]. Acetaldehyde is not a substrate for ALDH3A1, which is seemingly in line with the observation that ALDH3A1 protein is undetectable in the liver [91]. However, a recent report showed that pharmacological activation of ALDH3A1 facilitated acetaldehyde metabolism *in vivo*, suggesting that ALDH3A1 may contribute to cellular mechanisms that detoxify acetaldehyde [92]. A global *Aldh3a1* knockout (*Aldh3a1*$^{-/-}$) strain was originally generated by Nees and co-workers [93] and has been crossbred into B6 background [93]. *Aldh3a1*$^{-/-}$ mice in the mixed background develop cataracts at one month of age [76] and B6 *Aldh3a1*$^{-/-}$ mice display a phenotype of corneal haze [94]. Much like the *Aldh3a1*$^{-/-}$ mice, the *Aldh1a1/Aldh3a1* double knockouts (*Aldh1a1*$^{-/-}$/*Aldh3a1*$^{-/-}$) mice develop cataracts, but to a more severe extent [76]. As such, the majority of the research using these mouse strains has focused on elucidating the enzymatic and non-enzymatic functions of ALDH3A1 and ALDH1A1 in the cornea [95]. Given the recent experimental evidence, *Aldh3a1*$^{-/-}$ and *Aldh1a1*$^{-/-}$/*Aldh3a1*$^{-/-}$ strains may provide new insights into the interactive actions of these ALDH isozymes in alcohol metabolism and toxicities.

14.4.8 Aldh3a1C244A *and* Aldh1a1C303A *Knock-in Mice*

A large body of evidence supports the notion that the accumulation of high concentrations of ALDH3A1 and ALDH1A1 proteins in the cornea exert multifaceted functions involving both enzymatic and non-enzymatic properties of these proteins [91], the latter of which has been proposed to involve direct protein-protein interactions [96]. To elucidate the specific enzymatic and/or non-enzymatic functions of ALDH3A1 and ALDH1A1, our group has recently generated knock-in (KI) mouse lines for *Aldh3a1* and *Aldh1a1*, respectively. These strains were developed by introducing a Cys→Ala mutation at the catalytically-essential site of ALDH3A1 (codon 244) and ALDH1A1 (codon 303) proteins, respectively (unpublished work) and this results in the mice expressing enzymatically-inactive forms of these ALDH isozymes. These KI mouse strains may serve as additional models for alcohol research and are of particular utility for exploring non-enzymatic (or structural) functions of ALDH3A1 and ALDH1A1 in alcohol metabolism and toxicities.

14.5 Mouse Models with Glutathione Deficiency

It is well accepted that the generation of free radicals, such as ROS, during excessive consumption of ethanol depletes glutathione (GSH). It is believed that reductions in hepatic GSH levels are a key pathogenic event mediating ethanol-induced

liver injury [97–99]. Glutathione is a ubiquitous antioxidant and is essential for maintaining cellular redox homeostasis by detoxifying reactive xenobiotics and scavenging ROS derived from cellular metabolism. Due to its abundance in the liver, GSH plays a crucial role in defending against oxidative liver injuries. It is synthesized by two sequential reactions, the first and rate-limiting step of which is catalyzed by the enzyme glutamate-cysteine ligase (GCL). In higher eukaryotes, GCL comprises a catalytic (GCLC) and a modifier (GCLM) subunit. GCLC possesses all of the catalytic activity of GCL, and GCLM serves to optimize the kinetic properties of GCLC. Due to its essential role in GSH biosynthesis, GCL has been the principal target for generating animal models with GSH deficiency. Comprehensive reviews on GSH metabolism and functions and the GCL enzyme can be found elsewhere [100, 101].

14.5.1 Gclc *Conditional* (Gclc$^{f/f}$) *Knockout*

The global gene knockout of *Gclc* results in embryonic lethality, indicating that GSH is indispensable for early mouse development [102]. The *Gclc* floxed (*Gclc*$^{f/f}$) strain was developed and originally characterized by Chen and colleagues [103]. The pathophysiological role of GSH deficiency in hepatocytes was investigated using the hepatocyte-specific *Gclc* knockout (*Gclc*$^{h/h}$) mice created by intercrossing *Gclc*$^{f/f}$ and *Alb-Cre* mice [103]. *Gclc*$^{h/h}$ mice experience almost complete loss of hepatic GSH (~5% of normal) and die from acute liver failure when mitochondrial failure occurs [103]. Chronic administration of N-acetylcysteine, a treatment that promotes only a small increase in liver GSH levels (to 8% of normal), partially preserves the mitochondrial GSH pool and function, and allows *Gclc*$^{h/h}$ mice to survive to adulthood, albeit with the serious liver pathologies fibrosis and cirrhosis [104]. The *Gclc*$^{f/f}$ mice represent a unique model that can be used to elucidate cell-specific functions of GSH in ethanol metabolism and toxicity.

14.5.2 *Global* Gclm (Gclm$^{-/-}$) knockout

The *Gclm*$^{-/-}$ strain was developed and originally characterized by Yang and coworkers [79]. These mice are viable and fertile, despite having only 9–16% of the normal GSH levels in liver, lung, pancreas, erythrocytes, and plasma [105]. Except when challenged with oxidant stress [106, 107], *Gclm*$^{-/-}$ mice exhibit no overt phenotype, making them a useful model for studying the pathophysiological roles of chronic GSH depletion. Interestingly, these mice are protected against liver injuries induced by a variety of hepatic insults, including chronic ethanol treatment [101]. In addition, these mice are completely protected from ethanol -induced steatosis [108]. Thus, *Gclm*$^{-/-}$ mice represent an animal model wherein the effects of significant depletion of GSH on interventions, such as alcohol, can be examined.

14.5.3 Gclm *and* Nrf2 *Global Double-Knockout*

In examining the effects of GSH effects on alcohol metabolism, Nrf2 was shown to be upregulated upon ethanol treatment of *Gclm*$^{-/-}$ mice [108]. In a state of chronic GSH depletion, the Keap1-Nrf2-ARE signaling pathway appears necessary for the paradoxical resilience to ethanol-induced liver toxicity [109]. *Nrf2*$^{-/-}$ mice are highly sensitive to ethanol toxicity and show increased morbidity and mortality upon ethanol treatment [110]. Our group has cross-bred *Nrf2*$^{-/-}$ mice with the *Gclm*$^{-/-}$ mice and have preliminary data to suggest that the resultant *Nrf2*$^{-/-}$/*Gclm*$^{-/-}$ mice develop more serious hepatitis as they age than *Gclm*$^{-/-}$ or WT mice. *Nrf2*$^{-/-}$/*Gclm*$^{-/-}$ mice are viable and reach maturity; however, they do not produce progeny. This strain is an excellent model for exploring spontaneous development of hepatitis.

14.6 Concluding Remarks

Alcohol's deleterious effects have been implicated in various diseases, such as steatohepatitis, chronic liver disease, and cancers, such as those involving the mouth, liver, pancreas, colorectal region, and breast. Because alcohol metabolism is multifaceted, the genetically-engineered mouse models (discussed in this review) represent valuable tools that allow the further exploration of the mechanisms by which ethanol elicits its deleterious pathophysiological effects. Our diverse mouse model inventory contains a genetic variation for each step of the ethanol metabolic pathway, facilitating the way for the creation of novel therapeutic interventions to treat diseases associated with alcohol consumption.

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