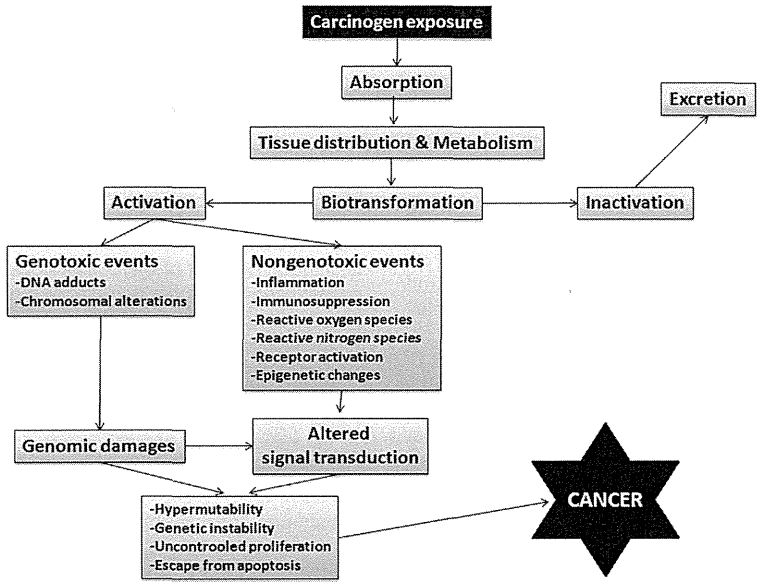

Figure 4 Metabolism and biological effects of genotoxic and nongenotoxic carcinogens on carcinogenesis.

that may prevent the entry of cells with DNA damage into the cell cycle prior to when DNA repair occurs and the cell divides.[53] The capacity of cancer cells to evade the cellular defense mechanism strongly contributes to carcinogenesis.

Alterations in the *ras* gene have been identified in several chemical-induced neoplasms in rodents. Mutations of the *ras* gene exist in about 20% of human neoplasms in the colon, breast, lung, and bladder.[54] An analysis of the *ras* gene isolated from the DNA of neoplasms revealed that changes in the sequence of nucleotides correspond to the places where carcinogens interact with DNA. Of note, each chemical compound appears to create its own unique fingerprint on the DNA.

The tumor suppressor genes, such as those encoding *p53*, *p21*, and *pRb*, play crucial roles in cellular protection, because they encourage the blockade of the cell cycle at the G1 phase.[55] The loss of the pRb protein function provokes an increase in the cell proliferation rate and an absence of terminal differentiation. *p53* can interrupt the cell cycle at G1 and allow cells to repair DNA damage.[37]

The most prominent and best-studied tumor suppressor is *p53*. When DNA is damaged, *p53* can induce either cell cycle arrest or apoptosis in order to maintain the stability of the cell's genome.[56] The loss of *p53* during carcinogenesis can predispose preneoplastic cells to accumulate additional mutations by blocking the normal apoptotic response to genetic damage.[35] The loss of *p53* function also activates proto-oncogenes and inactivates other tumor suppressor genes, and therefore has an integral role in chemical carcinogenesis.[27] The biological activity of p53 protein is largely related to its ability to bind transcriptional regulatory elements in the DNA. The search for critical genes regulated by *p53* led to the discovery of the gene encoding *p21*, which inhibits cyclin-dependent kinases, thus providing a functional link between *p53* and the cell cycle.[30]

The mismatch repair pathway is also influenced by the *p53* family. *p53* and *p73* induce the expression of p53R2, a gene that is homologous to the R2 regulatory subunit of ribonucleotide reductase.[57] p53R2 functions in a nonspecific manner to increase the pool of free dNTPs when the need for repair arises. Although p53R2 and R2 are similar, they differ in their N-terminal amino acid sequence and regulation. p53R2 is induced by p53 and p73, whereas R2 synthesis occurs during S phase. The p53R2 and R1 complex functions as an active ribonucleotide reductase.[58] p53 also upregulates two very important proteins in the mismatch repair pathway: human MutS homologue 2 (hMSH2) and proliferating cell nuclear antigen (PCNA).[59] Mutations of hMSH2 result in hereditary nonpolyposis colorectal cancer, a colorectal cancer syndrome. hMSH2 functions in mismatch recognition and binds mismatched bases.[60] PCNA, a cofactor for DNA polymerase δ is another p53 target gene that interacts with hMSH2 to facilitate hMSH2 transfer to mismatched bases.[61]

The genes involved in carcinogenesis are classified as caretakers and gatekeepers.[51,62] This classification is based on their involvement in maintaining the genomic integrity and DNA repair, respectively.[62] The caretakers are responsible for maintenance of the genome stability. Mutations in the caretaker genes, which are considered to be typical tumor suppressors, compromise the genome stability, and more specifically, increase the probability of mutation in the gatekeepers, which include both tumor suppressor genes and oncogenes.[63] Gatekeeper genes regulate neoplastic development by inhibiting the cell growth.[51] By contrast, the inactivity of caretaker genes does not support the induction of neoplasia, instead favoring genetic instability, which results in an increase in mutations across all genes, including the gatekeeper(s). Neoplasms with an inactive gatekeeper gene can progress quickly as a consequence of its effect on genes that directly control cell death.[51]

5. Proteomics in chemical carcinogenesis

Chemical carcinogenesis studies and, in consequence, biomarker discovery research, have usually placed their focus on the initiation part of the initiation/promotion model of carcinogenesis, which becomes apparent when looking at the impressive number of biomarker studies targeting genotoxic effects. However, exposure to some chemicals has been shown to result in carcinogenesis without involving the initiation step. The mechanism of nongenotoxic carcinogenesis is still incompletely understood, and an active debate continues regarding the relative contribution of procarcinogenic endogenous mechanisms, including the

generation of free radicals and the perturbation of epigenetic mechanisms by chemical carcinogens. The next critical step in carcinogenesis is the point when these altered cells start clonal expansion. It is important to identify and validate biomarkers indicating the start of clonal expansion. For this purpose, a proteomic analysis focusing on the effects of chemical carcinogens would be useful. Two-dimensional electrophoresis with subsequent matrix-assisted laser desorption and ionization time-of-flight mass spectrometry for protein separation and identification can be applied in these proteomic studies.[64]

Alterations of highly abundant proteins have been identified, which, irrespective of the wide differences in study design and technologies used, can be grossly assigned into three functional classes: (1) proteins related to the cellular stress response; (2) inflammation; and (3) stimulation of the immune system.[65] Of note, the observed protein alterations are not causal factors in the development of chemically induced cancer, but rather reflect common reactions to cellular perturbations. In order to gain deeper insights into the process of chemical carcinogenesis, the previously applied "shotgun" analyses have to be abandoned in favor of targeted proteomic approaches focusing on the accurate identification and quantification of selected proteins. Advanced analytical techniques, such as selective reaction monitoring and multiple reaction monitoring, may have the potential to contribute to the elucidation of chemical carcinogenesis.

6. MicroRNA

A number of recent studies have reported the involvement of microRNAs (miRNAs) in the regulation of cancer initiation, development, and metastasis.[66] In malignant cells, miRNAs are often dysregulated, with their expression patterns being correlated with clinically relevant tumor characteristics.[67] Several studies on the relationship between miRNAs and carcinogen exposure have also been reported.[68] These studies indicated that alterations in genes encoding miRNA genes play an important role in chemical carcinogenesis. A number of genotoxic carcinogens that dysregulate miRNA expression have been identified. The currently available information suggests that miRNA expression is associated with tumor initiation.[68,69] The expression of many miRNAs is readily changed in cells and target tissues after acute or chronic exposure to genotoxic carcinogens. Many of the differentially expressed miRNAs are involved in regulating genes that are important for carcinogen metabolism, DNA repair, apoptosis, and other cancer-related functions.

The progression phase of carcinogenesis is less well understood. During this phase, there is further growth and expansion of the tumor cells over that of normal cells. The genetic material of the tumor is thus more fragile and prone to additional mutations. These mutations occur in genes that regulate the growth and cell functions, such as oncogenes, tumor suppressor genes, and DNA mismatch-repair genes. These changes contribute to tumor malignancy. Because miRNAs can function as oncogenes or as tumor suppressor genes, miRNAs have been found to have a role in the progression of chemical-induced tumorigenesis.[68] Alterations in the miRNA expression in tumors induced by chemical carcinogens play an important role in tumor development.[68]

Therefore, the current evidence shows that miRNAs play important roles in every stage of chemical carcinogenesis, including initiation, promotion, and progression. Changes in the miRNA(s) occur prior to tumor formation, and are not merely a consequence of a transformed state. The expression of a large number of miRNAs is readily changed in the target tissues after acute or chronic exposure to carcinogens, but these changes are not observed in nontarget tissues or following exposure to noncarcinogenic chemicals. Many of the miRNAs deregulated by carcinogens are involved in regulating genes that are important for chemical carcinogenesis.

7. Conclusion and future perspectives

Chemical carcinogenesis has multiple stages and multifactorial processes, which are associated with genetic alterations. The acquisition of the capacity to survive and grow independently from other cells represents a crucial event in the process of cancer development. Most of the morphological, biochemical, and genetic changes should be considered to be a reflection of the adaptation of neoplastic cells to survive. The prediction of chemical carcinogenicity is of great importance for human risk assessment.

The research on chemical carcinogenesis has a rich history of scientific accomplishment that includes the fields of cancer biology, cancer risk assessment, public health policy, and an understanding of lifestyle- and occupation-related causes of cancer, as well as cancer chemoprevention. The gene–environment interactions and interindividual variations in the molecular epidemiology of human cancer risk are beginning to be understood based on studies of chemical carcinogenesis, cellular and molecular biology, and epidemiology. Based on these investigations of chemical carcinogenesis, many biomarkers of cancer risk and detection have been developed. These include carcinogen-DNA adducts, somatic mutations, and the mutation spectrum linking carcinogen exposure and DNA adduction with mutation.

Chemical carcinogens and viral interactions may have synergistic effects on cancer development: dietary AFB_1 and HBV infection results in the occurrence of hepatocellular cancer. Chemical carcinogenesis using rodent models has also played, and continuous to play, an important role in the field of cancer chemoprevention and in our understanding of the mechanisms of inflammation-associated cancer and the contribution of miRNAs to cancer. However, additional studies of chemical carcinogenesis related to stem cells and the epigenetic alterations that occur during chemical carcinogenesis are warranted to provide a better understanding of carcinogenesis and to gain an insight into better strategies to prevent, detect, and treat cancer.

Acknowledgments

Funding was provided from the Ministry of Health and Welfare (a Grant-in-Aid for the 2nd and 3rd Terms Comprehensive 10-Year Strategy for Cancer Control) and the Ministry of Education, Science, Sports, and Culture (Grants-in-Aid, Nos. 13671986 and 23501324) of Japan.

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(–)-Epigallocatechin-3-gallate suppresses hepatic preneoplastic lesions developed in a novel rat model of non-alcoholic steatohepatitis

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Abstract

Purpose: Non-alcoholic fatty liver disease (NAFLD) ranges from simple steatosis to non-alcoholic steatohepatitis (NASH). NASH, which is accompanied by increased oxidative stress and inflammation in the liver, is associated with hepatic carcinogenesis. Green tea catechins (GTCs) possess anti-oxidant, anti-inflammatory, and cancer-preventive properties. In this study, we investigated whether (–)-epigallocatechin-3-gallate (EGCG), a major component of GTCs, inhibits NAFLD/NASH-related liver tumorigenesis.

Methods: Male 8-week-old Sprague–Dawley (SD) rats were administered a single intraperitoneal injection of a hepatic carcinogen diethylnitrosamine (DEN, 30 mg/kg body weight) and then fed a high-fat diet (HFD) for 7 weeks. The rats were also provided tap water containing 0.01% or 0.1% EGCG during the experiment.

Results: At sacrifice, the livers of SD rats treated with DEN and HFD exhibited marked development of glutathione *S*-transferase placental form (GST-P)-positive foci, a hepatic preneoplastic lesion, and this was associated with hepatic steatosis, oxidative stress and inflammation, and hepatocyte proliferation. EGCG administration, however, inhibited the development of GST-P-positive foci by decreasing hepatic triglyceride content, reducing hepatic fibrosis, lowering oxidative stress, attenuating inflammation, and inhibiting excessive hepatocyte proliferation in DEN- and HFD-treated SD rats. These findings suggest that the experimental model of SD rats treated with HFD and DEN, in which histopathological and pathophysiological characteristics of NASH and the development of hepatic premalignant lesions were observed, might facilitate the evaluation of liver tumorigenesis associated with NAFLD/NASH.

Conclusions: Administering EGCG, a GTC, might serve as an effective chemoprevention modality for NAFLD/NASH-related liver tumorigenesis.

Keywords: Non-alcoholic steatohepatitis; Liver tumorigenesis; Oxidative stress; Inflammation; EGCG

Background

Non-alcoholic fatty liver disease (NAFLD), which is considered a hepatic manifestation of the metabolic syndrome, is currently one of the most common chronic liver diseases worldwide. NAFLD is classified into simple steatosis and non-alcoholic steatohepatitis (NASH), the latter being a severe condition of inflamed fatty liver that can, in time, lead to hepatic fibrosis, cirrhosis, hepatocellular carcinoma (HCC) development, and result in increased mortality (Chiang et al., 2011; Cusi, 2012; Siegel and Zhu, 2009). Steatohepatitis has been indicated by epidemiological studies to be a significant risk factor for developing HCC, at an annual HCC incidence of 2%–3% in patients with NASH (Adams et al., 2005; Ascha et al., 2010). In 1998, Day and James proposed a "two-hit theory" to explain NAFLD/NASH pathogenesis: the first hit, hepatic steatosis, increases the sensitivity of the liver to proinflammatory insults, while the second hit involves oxidative stress (Day and James, 1998). Oxidative

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stress, which is associated with HCC development (Suzuki et al., 2013), is substantially higher in NASH patients than in NAFLD patients and normal control subjects (Sanyal et al., 2001). Moreover, tumor necrosis factor (TNF)-α and interleukin (IL)-6, both of which are major proinflammatory cytokines, play a critical role in obesity-related steatohepatitis and subsequent liver tumorigenesis (Park et al., 2010).

Several animal models that mimic the pathophysiological mechanisms associated with NAFLD/NASH-related liver carcinogenesis have been developed recently (Hebbard and George, 2011; Schattenberg and Galle, 2010; Kochi et al., 2013). For instance, *db/db* mice, which exhibit obesity, diabetes, dyslipidemia, and severe steatosis, are susceptible to liver tumorigenesis induced by a hepatic carcinogen diethylnitrosamine (DEN), and thus are regarded as useful animal models for investigating pathobiology of NAFLD/NASH-related liver carcinogenesis and for screening the chemopreventive effects of various compounds either synthetic or natural on NAFLD/NASH-related liver carcinogenesis (Iwasa et al., 2010; Shimizu et al. 2011a, 2011b, 2011c). Recently, Wang et al. (Wang et al. 2009) reported that NASH induced by a high-fat diet (HFD) promoted DEN-initiated early hepatocarcinogenesis in Sprague–Dawley (SD) rats and that this was associated with increased oxidative stress and inflammation. This animal model is also useful for investigating the efficacy of certain types of synthetic compounds and/or phytochemicals in preventing NASH-promoted liver carcinogenesis (Wang et al., 2010).

The prevalence of NAFLD/NASH has risen recently in parallel with the dramatic increase in obesity and its related metabolic abnormalities, especially diabetes mellitus (Chiang et al., 2011; Cusi, 2012; Siegel and Zhu, 2009), indicating an urgent requirement for developing an effective strategy to treat NAFLD/NASH and, consequently, to prevent NAFLD/NASH-related liver carcinogenesis. A recent randomized trial (Sanyal et al., 2010) has shown that treatment with vitamin E, an antioxidant, reduces steatosis and lobular inflammation in the liver of NASH patients. Reducing oxidative stress and inhibiting the inflammation induced by obesity and steatosis are also effective in preventing obesity- and diabetes-related hepatotumorigenesis (Shimizu et al. 2013, 2012). These reports suggest that targeting oxidative stress and chronic inflammation is an optimal strategy for preventing NAFLD/NASH-related liver carcinogenesis.

Green tea catechins (GTCs) might be one of the most promising candidate compounds for preventing NAFLD/NASH-related liver carcinogenesis because they are considered to protect against metabolic disorders such as NAFLD (Masterjohn and Bruno, 2012; Thielecke and Boschmann, 2009) and also display cancer chemopreventive properties in various tissues, including the liver (Shimizu et al. 2011b; Kochi et al., 2013; Yang et al., 2009). In this study, we developed a novel rat model of NAFLD/NASH-related carcinogenesis and investigated the potential capacity of (−)-epigallocatechin-3-gallate (EGCG), a major component of GTCs, to inhibit the occurrence of HFD- and DEN-induced glutathione *S*-transferase placental form (GST-P)-positive foci, an indicator of preneoplastic HCC lesions in rats (Tsuda et al., 2003; Ando et al., 2007).

Results

General observations

At the end of the experimental period, the rats in the 3 groups exhibited no significant differences with respect to the mean body, liver, and kidney weights (Table 1). Histopathological examination revealed that administering EGCG produced no detectable toxic effects on critical organs including the liver, kidney, and spleen (data not shown).

Effects of EGCG on hepatic steatosis and serum ALT levels in rats

At sacrifice, macrovesicular steatosis with ballooned hepatocytes, Mallory-Denk bodies, and foci of inflammatory cells, which are a recognized feature of NASH (Kleiner et al., 2005), were observed in the livers of rats in all groups, indicating that the histopathological characteristics that develop in this animal model reproduce those of NASH. However, these pathological effects were alleviated by the administration of 0.01% and 0.1% EGCG (Figure 1a and 1b). In particular the total NAFLD activity score (NAS) consisting of the steatosis, inflammation, and ballooning scores, was significantly lower in 0.1% EGCG-treated rats than in EGCG-untreated control rats (Figure 1c, $P < 0.05$). Similar results were obtained following measurement of intrahepatic lipid content: the levels of liver triglycerides in

Table 1 Body and organ weights of the experimental rats

Group No.	EGCG	No. of rats	Body weight (g)	Relative organ weight (g/100 g body weight)	
				Liver	Kidneys
1	-	7	512.7 ± 37.8[a]	3.8 ± 0.5	0.6 ± 0.4
2	0.01%	6	495.5 ± 29.0	3.9 ± 0.6	0.7 ± 0.3
3	0.1%	6	501.6 ± 44.3	3.5 ± 0.2	0.6 ± 0.3

[a]Mean ± SD.

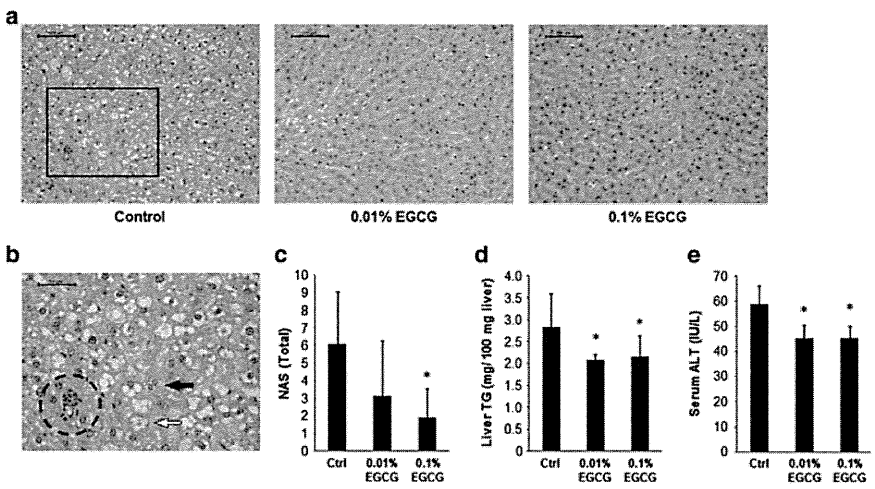

Figure 1 Hepatic histopathology, intrahepatic levels of triglycerides, and serum levels of ALT. (a) and **(b)** H&E staining of liver sections from the experimental rats. **(a)** Representative photomicrographs of liver sections of rats treated DEN/HFD with or without EGCG. Bars, 100 μm. **(b)** An enlarged photo of the liver section from a rat treated DEN/HFD without EGCG (an enclosed area with square in Figure 1a). Note ballooned hepatocytes (indicated by the white arrow), Mallory-Denk bodies (indicated by the black arrow), and focus of inflammatory cells (circled by dotted line). Bars, 50 μm. **(c)** The NAS was determined from histopathological analysis. **(d)** Hepatic lipids were extracted from frozen liver samples of rats in the 3 groups, and the triglyceride (TG) levels were measured. **(e)** Blood samples were collected at sacrifice and the serum levels of alanine aminotransferase (ALT) were determined. The values are expressed as mean ± SD. *$P < 0.05$. Ctrl, control group.

the DEN- and HFD-treated SD rats were significantly decreased when they received 0.01% and 0.1% EGCG instead of water (Figure 1d, $P < 0.05$). The serum alanine aminotransferase (ALT) levels were also significantly decreased with EGCG treatment at both concentrations relative to the levels in the water-treated group (Figure 1e, $P < 0.05$), indicating that EGCG protected against hepatic steatosis and subsequent hepatocyte injury induced by DEN and HFD.

Effects of EGCG on liver fibrosis in rats

Examination of Azan-stained liver sections revealed that DEN- and HFD-treated SD rats developed perisinusoidal fibrosis, but that EGCG administration reduced liver fibrosis in the animals (Figure 2a). The liver fibrosis score was also significantly decreased by EGCG administration (Figure 2b). Furthermore, EGCG treatment significantly lowered (relative to control) the hepatic levels of TIMP-1 and TIMP-2 mRNA in the DEN- and HFD-treated SD rats (Figure 2c, $P < 0.05$).

Effects of EGCG on the development of hepatic preneoplastic lesions in rats

At the end of the experiment, GST-P-positive foci were detected in the livers of the rats, all of which had received DEN/HFD (Figure 3a). However, compared with rats in the control group that were not treated with EGCG, rats treated with EGCG showed a significant reduction in the number of GST-P-positive foci: 86% and 87% reduction relative to control following treatment with 0.01% and 0.1% EGCG, respectively (Figure 3b, $P < 0.01$).

Effects of EGCG on oxidative stress in rats

Oxidative stress plays a critical role in the NAFLD-to-NASH progression and HCC development (Chiang et al., 2011; Cusi, 2012; Siegel and Zhu, 2009). Therefore, we examined the levels of oxidative stress and antioxidant biomarkers in the experimental rats. Rats administrated with 0.1% EGCG had significantly reduced levels of urinary 8-OHdG, a marker of DNA damage induced by oxidative stress, compared with EGCG-untreated control rats (Figure 4a, $P < 0.01$). The serum d-ROM level, which reflects serum hydroperoxide levels, was also decreased relative to control in rats treated with 0.01% and 0.1% EGCG (Figure 4b, $P < 0.01$). Moreover, the antioxidant enzyme (catalase and GPx-1) levels were significantly increased in the livers of rats that received EGCG treatment (Figure 4c, $P < 0.05$). These results indicate that drinking EGCG attenuated both systemic and hepatic oxidative stress in our rat model of NAFLD/NASH-related liver tumorigenesis.

Effects of EGCG on hepatic expression of TNF-α, IL-6, and IL-1β mRNA in rats

Chronic inflammation is implicated in the progression of NASH and subsequent liver carcinogenesis (Chiang et al., 2011; Cusi, 2012; Siegel and Zhu, 2009; Park et al., 2010). Therefore, the mRNA expression levels of 3 inflammatory

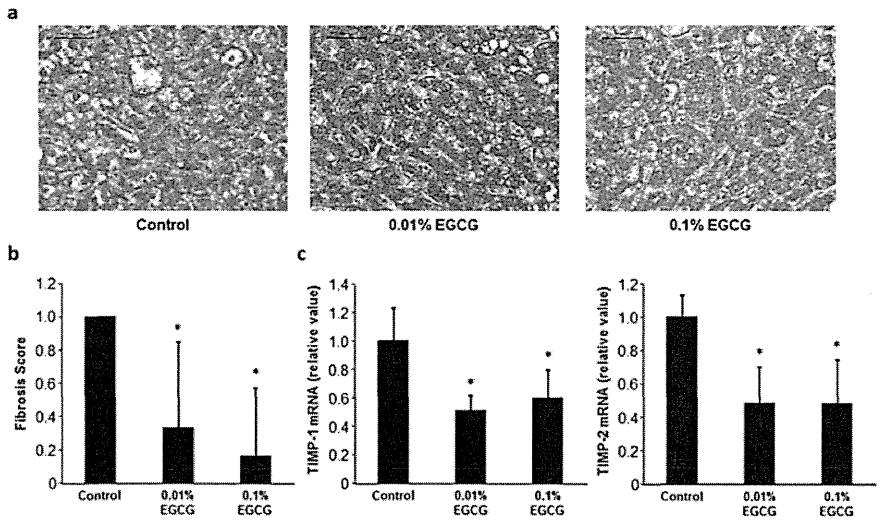

Figure 2 Liver fibrosis and hepatic expression levels of TIMP-1 and TIMP-2 mRNA. (a) Representative photomicrographs of Azan staining of liver tissues to show fibrosis. Bars, 50 μm. Note reduction of liver fibrosis (blue) by the treatment with EGCG. **(b)** Liver fibrosis evaluation was based on the NAS system. **(c)** After total RNA was isolated from the livers of the rats, the levels of TIMP-1 and TIMP-2 mRNA were measured using specific primers and quantitative real-time RT-PCR. The values are expressed as mean ± SD. * $P < 0.05$.

mediators, TNF-α, IL-6, and IL-1β, were measured in the livers of DEN- and HFD-treated SD rats. As shown in Figure 5, quantitative real-time RT-PCR analysis revealed that rats that received 0.1% EGCG exhibited significantly lower hepatic levels of TNF-α ($P < 0.05$), IL-6 ($P < 0.01$), and IL-1β ($P < 0.05$) mRNA than control rats that received only water, and the hepatic levels of IL-6 mRNA were also decreased by the administration of a low dose (0.01%) of EGCG ($P < 0.01$).

Effects of EGCG on hepatocyte proliferation and hepatic expression of cyclin D1 mRNA in rats

The PCNA-labeling index of non-lesional hepatocytes in DEN- and HFD-treated SD rats was determined based on the findings of immunohistochemical examination of sections (Figure 6a). The mean PCNA-labeling indices measured for rats administered 0.01% and 0.1% EGCG were significantly lower than that for EGCG-untreated control rats (Figure 6b, $P < 0.01$). Furthermore, the levels

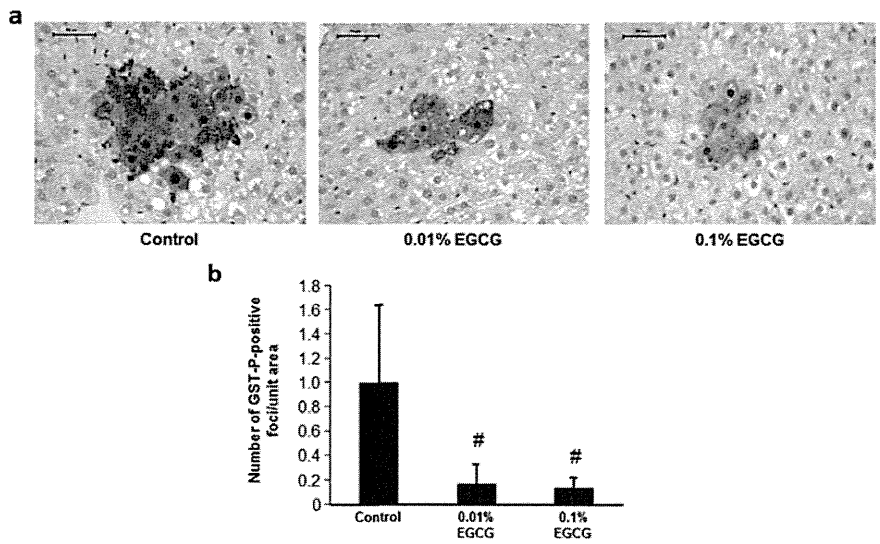

Figure 3 The development of GST-P-positive foci in the livers. (a) Representative photomicrographs of GST-P-positive foci. Note a large GST-P focus in a rat treated with DEN/HFD. Bars, 50 μm. **(b)** The average numbers of GST-P-positive foci formed in the livers of the rats in the 3 groups. The values are expressed as mean ± SD. # $P < 0.01$.

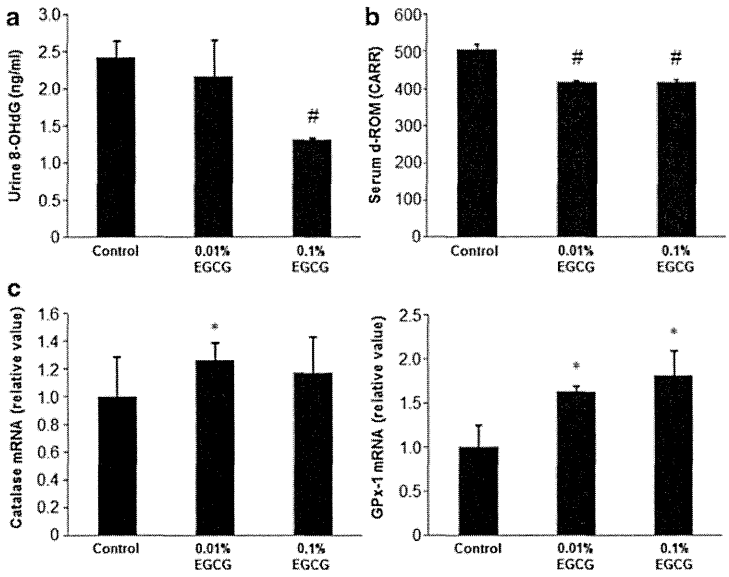

Figure 4 Urinary 8-OHdG and serum d-ROM levels and on the hepatic catalase and GPx-1 mRNA levels. (a) The urinary 8-OHdG levels were measured using ELISA. **(b)** Hydroperoxide levels in the serum were determined using the d-ROM test. **(c)** Levels of catalase and GPx-1 mRNA in the livers of the experimental rats were measured using specific primers and quantitative real-time RT-PCR. The values are expressed as mean ± SD. #*P* <0.01, **P* <0.05.

of cyclin D1 mRNA in liver were also markedly decreased in EGCG-treated rats relative to that in control rats (Figure 6c, $P < 0.05$), indicating that EGCG significantly inhibited hepatocyte proliferation in DEN- and HFD-treated SD rats.

Discussion

An increase in the prevalence of NAFLD/NASH, which can progress to HCC, is a major healthcare problem worldwide (Chiang et al., 2011; Cusi, 2012; Siegel and Zhu, 2009). Therefore, developing an effective strategy for preventing NAFLD/NASH-related liver tumorigenesis is critical for improving the prognosis of patients with these diseases. The results of this study clearly indicate that EGCG, a GTC, effectively prevents the development of hepatic preneoplastic lesions, which manifest as GST-P-positive foci, in our rat model of NAFLD/NASH-related liver tumorigenesis. The rodent model used in this study, which was modified from a model described previously (Wang et al., 2009), reflects the pathological alterations implicated in NAFLD/NASH and NAFLD/NASH-related liver tumorigenesis, including the induction of oxidative stress and chronic inflammation (Chiang et al., 2011; Cusi, 2012; Siegel and Zhu, 2009; Park et al., 2010). Therefore, we consider the present model to be an appropriate and a useful animal model for analyzing the mechanisms of NAFLD/NASH-related liver tumorigenesis and for evaluating the efficacy of specific chemopreventive agents that can suppress such tumorigenesis.

Among the numerous pathophysiological conditions associated with NAFLD/NASH, oxidative stress is regarded as one of the key mechanisms for the development of

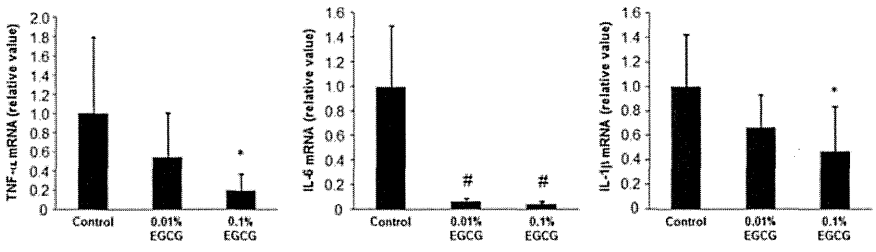

Figure 5 Hepatic expression of TNF-α, IL-6, and IL-1β mRNA. Total RNA was isolated from the livers of rats in the 3 groups and the expression levels of TNF-α, IL-6, and IL-1β mRNA were determined using specific primers and quantitative real-time RT-PCR. The values are expressed as mean ± SD. # *P* <0.01, **P* <0.05.

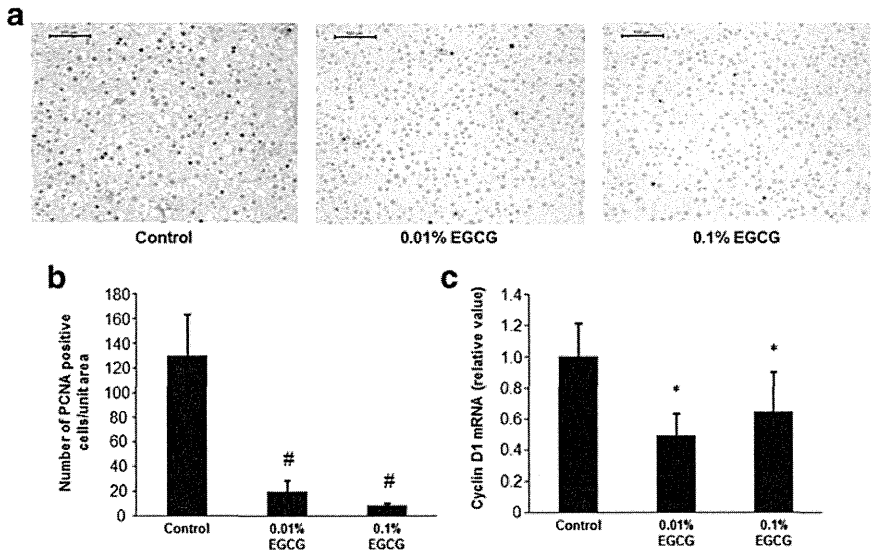

Figure 6 Hepatocyte proliferation and hepatic expression of cyclin D1 mRNA. (a) Immunohistochemical labeling for PCNA in the livers of the experimental rats. Bars, 100 μm. **(b)** The PCNA-labeling index in non-lesional hepatocytes was determined by counting the hepatocytes with PCNA-positive nuclei to calculate their percentage in the hepatocyte population. **(c)** The cyclin D1 mRNA levels in the liver of the experimental rats were determined using specific primers and quantitative real-time RT-PCR. The values are expressed as mean ± SD. # $P < 0.01$, *$P < 0.05$.

HCC. In the progression of NAFLD to NASH, an increase in oxidative stress, which is defined as the overproduction of reactive oxygen species combined with inadequate anti-oxidative defense mechanisms, produces DNA damage and gene mutations associated with liver carcinogenesis (Chiang et al., 2011; Cusi, 2012; Siegel and Zhu, 2009; Park et al., 2010). Conversely, treatment with antioxidants such as vitamin E can reduce hepatic steatosis, lobular inflammation, and serum ALT levels, as shown by a clinical trial conducted in NASH patients (Sanyal et al., 2010). Administering pentoxifylline, which is known to decrease oxidative stress and inhibit TNF-α expression, also improved the histological features of NASH in a recent randomized placebo-controlled trial (Zein et al., 2011). In this study, treatment with EGCG lowered the levels of oxidative stress-associated markers such as urinary 8-OHdG and serum d-ROM, whereas elevated the mRNA levels of two antioxidant enzymes, catalase and GPx-1, in the liver of DEN- and HFD-treated SD rats. These findings suggest that EGCG suppressed NAFLD/NASH-related liver tumorigenesis at least partly by reducing systemic and hepatic oxidative stress. Our results are consistent with those of previous studies showing that GTCs can protect against both oxidative stress and the progression of NAFLD to NASH (Park et al., 2011; Ueno et al., 2009; Kuzu et al., 2008).

Besides oxidative stress, chronic inflammation is critically involved in NAFLD/NASH-related liver tumorigenesis (Chiang et al., 2011; Cusi 2012; Siegel and Zhu, 2009; Park et al., 2010). Among the proinflammatory cytokines related to the progression of NASH, TNF-α and IL-6 play a pivotal role in hepatocyte injury and inflammation, which increase HCC risk (Park et al., 2010). Therefore, targeting TNF-α and IL-6 might be an effective method to suppress NAFLD/NASH-related liver tumorigenesis. GTCs are widely recognized to exert cancer-preventive effects partly by inhibiting the expression of TNF-α and IL-6 (Shimizu et al. 2011b; Kochi et al., 2013; Shirakami et al. 2008), indicating that the suppression of inflammation is one of the key mechanisms by which GTCs prevent cancer development. In this study, we found that mRNAs encoding TNF-α, IL-6, and IL-1β were expressed at significantly lower levels in the livers of EGCG-treated rats compared to that in EGCG-untreated rats. Therefore, in agreement with previous reports (Shimizu et al. 2011b; Kochi et al., 2013; Shirakami et al. 2008), the results of this study suggest that EGCG consumption suppressed the development of GST-P-positive foci in DEN- and HFD-treated SD rats by attenuating chronic inflammation.

In addition to reducing oxidative stress and chronic inflammation, both of which are secondary manifestations of NASH, administering EGCG improved hepatic steatosis, a primary manifestation of NASH (Day and James, 1998), decreased serum ALT levels, ameliorated liver fibrosis, and inhibited excessive hepatocyte proliferation in this study. GTCs have been demonstrated to attenuate hepatic fat accumulation in several laboratory animal studies (Kuzu et al., 2008; Kochi et al., 2013; Shimizu et al. 2011b; Park et al., 2011; Ueno et al., 2009);

these reports combined with the findings of this study are of interest because hepatic steatosis *per se* can induce hepatocyte proliferation and hepatic hyperplasia, both of which initiate the hepatic neoplastic process by increasing hepatocyte proliferative activity (Yang et al., 2001). Moreover, hepatic steatosis is critically related to liver fibrosis, which is a strong risk factor for the development of HCC (Powell et al., 2005). Therefore, suppression of hepatic steatosis and fibrosis by EGCG treatment might help to inhibit the progression of NAFLD/NASH-related liver tumorigenesis at an early stage.

Because GST-P-positive foci are generally accepted to be precursors or preneoplastic lesions of HCC in rats (Tsuda et al., 2003; Ando et al., 2007), the rodent model used in this study appears to be well suited for screening reagents that can prevent NAFLD/NASH-related liver tumorigenesis. However, the current study has one limitation that hepatocellular neoplasms, including HCC, did not develop within the experimental period. Because the duration of the experiment (7 weeks) might have been insufficient for the development of hepatic neoplasms, future studies should be conducted using longer-term experiments to confirm that HFD- and DEN-treated SD rats develop hepatocellular neoplasms.

Finally, we emphasize again that targeting metabolic abnormalities, especially oxidative stress and chronic inflammation, might be one of the most practical approaches for treating NAFLD/NASH and preventing NAFLD/NASH-related liver carcinogenesis (Shimizu et al. 2013, 2012). We consider GTCs including EGCG to be potentially effective and key candidates for this purpose, because these agents can target metabolic abnormalities and thus prevent relevant tumorigenesis, as shown by the results of this study and those from previous reports (Shimizu et al. 2011b; Kochi et al., 2013; Shimizu et al. 2008b; Thielecke and Boschmann, 2009; Grove and Lambert, 2010). Recent clinical trials have also demonstrated that GTC supplementation potently prevents the development of both colorectal adenomas and prostate cancers without causing adverse effects (Shimizu et al. 2008a; Bettuzzi et al., 2006). These beneficial effects of GTCs reported in clinical trials strongly encourage the clinical usage of GTCs for treating NAFLD/NASH patients to prevent the metabolic abnormalities, such as steatosis, hyperlipidemia, and hyperinsulinemia, as well as liver carcinogenesis.

Conclusions

In conclusion, administering EGCG effectively suppresses the early stage of hepatocarcinogenesis in our rat model of NAFLD/NASH by attenuating oxidative stress and chronic inflammation. Application of GTCs represents a potential new strategy for preventing the development of hepatic neoplasms in NAFLD/NASH patients.

Methods

Animals and chemicals

Male 7-week-old SD rats were obtained from Japan SLC, Inc. (Shizuoka, Japan) and humanely maintained at Gifu University Life Science Research Center in accordance with the Institutional Animal Care Guidelines. DEN was purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). HFD-60 (HFD, 506.2 kcal/100 g) with 62.2% of the calories derived from fat was purchased from Oriental Yeast (Tokyo, Japan) (Table 2). EGCG was obtained from Mitsui Norin Co. Ltd. (Tokyo, Japan).

Experimental procedure

The present study was approved by the Experimental Animal Research Committee of Gifu University. After 1-week-acclimatization with regular chow, all rats (n = 19) received a single intraperitoneal injection of DEN (30 mg/kg body weight) and were then randomly divided into 3 groups. Following DEN injection, the rats in Groups 2 and 3 (n = 6 in both groups) were provided tap water containing 0.01% or 0.1% EGCG, respectively, whereas the rats in Group 1 (n = 7) were provided tap water throughout the experiment, which lasted 7 weeks. All rats were fed a pelleted HFD throughout the experiment after DEN injection. At the end of the experiment, the 15-week-old rats were sacrificed by CO_2 asphyxiation and the development of GST-P-positive foci was evaluated. The concentration of EGCG used (0.1%), which was established based on the findings of previous chemopreventive studies

Table 2 Composition and calories of the experimental diet HFD-60

Ingredients	(g/kg diet)
Casein	256.0
Corn starch	160.0
Sucrose	55.0
Dextrose	60.0
Cellulose	66.1
Soybean oil	20.0
Lard	330.0
Vitamin mixture	35.0
Mineral mixture	10.0
Calcium carbonate	1.8
L-cysteine	3.6
Choline bitartrate	2.5
Energy	**(kcal/kg)**
	5062
	(%)
Protein	18.2
Fat	62.2
Carbohydrate	19.6

(Shimizu et al. 2011b; Kochi et al., 2013; Shirakami et al., 2008; 2009), was, in terms of units per body weight, within the physiological range measured in humans after daily intake of GTCs (Wang et al., 1991). Previously, GST-P-positive foci were markedly induced by DEN injection in HFD fed rats, but not in rats fed a normal diet (Wang et al. 2009), therefore we did not use a control group that was fed normal chow diet after DEN injection in the present study.

Histopathological examination and immunohistochemical analyses for GST-P and proliferating cell nuclear antigen (PCNA)

Maximum sagittal sections of 3 liver sublobes (central, lateral, and right-anterior) were used for histopathological examination. Formalin-fixed and paraffin-embedded livers were stained with hematoxylin & eosin (H&E) for conventional histopathology or with Azan stain to detect liver fibrosis. The histological features of the livers were evaluated using the NAFLD activity score (NAS) system (Kleiner et al., 2005), and the development of liver fibrosis was determined as described previously (Kleiner et al., 2005). Immunohistochemistry for GST-P (Ando et al., 2007) and PCNA (Iwasa et al., 2010) was performed using primary antibodies against GST-P (MBL Co. Ltd., Nagoya, Japan) and PCNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), respectively. The number of GST-P-positive foci, which was set as 3 or more positive cells (Kochi et al., 2013), was assessed per unit area (per cm^2). In the PCNA-immunostained sections, cells with intensely stained nuclei were considered to be positive for PCNA, and the indices (% PCNA-positive) were determined by counting at least 500 hepatocytes in each section (total of 3000 hepatocytes per rat) (Iwasa et al., 2010).

RNA extraction and quantitative real-time RT-PCR analysis

Total RNA was isolated from the livers of the rats by using RNeasy Mini kit (QIAGEN, Venlo, Netherlands) with on-column DNase I-digestion (Terakura et al., 2012). From 0.2 μg of total RNA, cDNAs were amplified using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Santa Clara, CA, USA) and an automated thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). Quantitative real-time reverse transcription-PCR (RT-PCR) analysis was performed using specific primers that amplify the TNF-α, IL-1β, IL-6, tissue inhibitor of metalloproteinases (TIMP)-1, TIMP-2, glutathione peroxidase (GPx)-1, catalase, cyclin D1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes. The sequences of these primers, which were obtained using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), are shown in Table 3. Each sample was analyzed on a LightCycler Nano (Roche Diagnostics, Mannheim, Germany) with FastStart Essential DNA Green Master (Roche Diagnostics). GAPDH amplified in parallel served as the internal control.

Table 3 Primer sequences

Target gene	Direction	Primer sequences (5′-3′)
Catalase	Forward	GCGAATGGAGAGGCAGTGTAC
	Reverse	GAGTGACGTTGTCTTCATTAGCACTG
Cyclin D1	Forward	TTCGTGGCCTCTAAGATGAAGG
	Reverse	TGAGCTTGTTCACCAGAAGCAG
Gapdh	Forward	AGTGCCAGCCTCGTCTCATAG
	Reverse	CCTTGACTGTGCCGTTGAACT
Gpx-1	Forward	GCTCACCCGCTCTTTACCTT
	Reverse	GATGTCGATGGTGCGAAAGC
Il-1β	Forward	AGGCTTCCTTGTGCAAGTGT
	Reverse	TCCTGGGGAAGGCATTAGGA
Il-6	Forward	CACTTCACAAGTCGGAGGCT
	Reverse	AGCACACTAGGTTTGCCGAG
Timp-1	Forward	ACAGCTTTCTGCAACTCGGA
	Reverse	AGTTTGCAAGGGATGGCTGA
Timp-2	Forward	TGGGAACGTGCATTTTGCAG
	Reverse	AAACACTGGTTGGAGGGCAA
Tnf-α	Forward	CCAGACCCTCACACTCAGATCA
	Reverse	TCCGCTTGGTGGTTTGCTA

Clinical chemistry

At sacrifice, the serum levels of ALT were measured using a standard clinical automatic analyzer (Type 7180; Hitachi, Tokyo, Japan).

Hepatic lipid analysis

Total lipids were extracted from approximately 200 mg of liver tissue (frozen at sacrifice) for each rat, and the triglyceride levels were measured using the triglyceride E-test kit (Wako, Osaka, Japan) (Iwasa et al., 2010).

Oxidative stress analysis

Urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels were determined using an ELISA kit (NIKKEN SEIL, Shizuoka, Japan). Serum levels of hydroperoxide, one of the markers for oxidative stress, were determined using the derivatives of reactive oxygen metabolites (d-ROM) test (FREE Carpe Diem; Diacron s.r.l., Grosseto, Italy) (Kochi et al., 2013).

Statistical analysis

All data are expressed as mean ± SD, and one-way analysis of variance (ANOVA) was used for comparisons between groups. If ANOVA indicated significant differences, the Tukey-Kramer test for multiple comparisons was performed to compare the mean values among the groups.

The differences were considered significant when the two-sided *P* value was less than 0.05. All analyses were conducted by the GraphPad InStat software, Version 3.05 (GraphPad Software; San Diego, CA, USA).

Abbreviations

ALT: Alanine aminotransferase; ANOVA: Analysis of variance; Ctrl: Control; DEN: Diethylnitrosamine; d-ROM: Derivatives of reactive oxygen metabolites; EGCG: (−)-epigallocatechin-3-gallate; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GPx: Glutathione peroxidase; GST-P: Glutathione *S*-transferase placental form; GTC: Green tea catechin; HCC: Hepatocellular carcinoma; HFD: High-fat diet; IL: Interleukin; NAFLD: Non-alcoholic fatty liver disease; NAS: NAFLD activity score; NASH: Non-alcoholic steatohepatitis; 8-OHdG: 8-hydroxy-2'-deoxyguanosine; RT-PCR: Reverse transcription-PCR; SD: Sprague–Dawley; TG: Triglyceride; TIMP: Tissue inhibitor of metalloproteinase; TNF: Tumor necrosis factor.

Competing interest

The authors declare that they have no competing interest.

Authors' contributions

TS made substantial contributions to conception and carried out the molecular biological studies. YS participated in the design of the study and carried out the molecular biological studies. MS participated in the design of the study and involved in drafting the manuscript and revising. TK carried out the molecular biological studies and made contributions to acquisition of data. TO made contributions to analysis of data. MK made contributions to analysis of data. MS involved in drafting the manuscript and revising. HT involved in drafting the manuscript and revising. TT carried out the pathological studies and performed the statistical analysis. HM made substantial contributions to analysis and interpretation of data and involved in drafting the manuscript and revising. All authors read and approved the final manuscript.

Acknowledgments

This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (No. 22790638 and No.25460988), Grant-in-Aid for the 3rd Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labor and Welfare of Japan, and Takeda Science Foundation.

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Received: 26 October 2013 Accepted: 21 December 2013
Published: 27 December 2013

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Cite this article as: Sumi *et al.*: (−)-Epigallocatechin-3-gallate suppresses hepatic preneoplastic lesions developed in a novel rat model of non-alcoholic steatohepatitis. *SpringerPlus* 2013 2:690.

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The Effect of Acyclic Retinoid on the Metabolomic Profiles of Hepatocytes and Hepatocellular Carcinoma Cells

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Abstract

Background/Purpose: Acyclic retinoid (ACR) is a promising chemopreventive agent for hepatocellular carcinoma (HCC) that selectively inhibits the growth of HCC cells (JHH7) but not normal hepatic cells (Hc). To better understand the molecular basis of the selective anti-cancer effect of ACR, we performed nuclear magnetic resonance (NMR)-based and capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS)-based metabolome analyses in JHH7 and Hc cells after treatment with ACR.

Methodology/Principal Findings: NMR-based metabolomics revealed a distinct metabolomic profile of JHH7 cells at 18 h after ACR treatment but not at 4 h after ACR treatment. CE-TOFMS analysis identified 88 principal metabolites in JHH7 and Hc cells after 24 h of treatment with ethanol (EtOH) or ACR. The abundance of 71 of these metabolites was significantly different between EtOH-treated control JHH7 and Hc cells, and 49 of these metabolites were significantly down-regulated in the ACR-treated JHH7 cells compared to the EtOH-treated JHH7 cells. Of particular interest, the increase in adenosine-5′-triphosphate (ATP), the main cellular energy source, that was observed in the EtOH-treated control JHH7 cells was almost completely suppressed in the ACR-treated JHH7 cells; treatment with ACR restored ATP to the basal levels observed in both EtOH-control and ACR-treated Hc cells (0.72-fold compared to the EtOH control-treated JHH7 cells). Moreover, real-time PCR analyses revealed that ACR significantly increased the expression of pyruvate dehydrogenase kinases 4 (PDK4), a key regulator of ATP production, in JHH7 cells but not in Hc cells (3.06-fold and 1.20-fold compared to the EtOH control, respectively).

Conclusions/Significance: The results of the present study suggest that ACR may suppress the enhanced energy metabolism of JHH7 cells but not Hc cells; this occurs at least in part via the cancer-selective enhancement of PDK4 expression. The cancer-selective metabolic pathways identified in this study will be important targets of the anti-cancer activity of ACR.

Citation: Qin X-Y, Wei F, Tanokura M, Ishibashi N, Shimizu M, et al. (2013) The Effect of Acyclic Retinoid on the Metabolomic Profiles of Hepatocytes and Hepatocellular Carcinoma Cells. PLoS ONE 8(12): e82860. doi:10.1371/journal.pone.0082860

Editor: Matias A. Avila, University of Navarra School of Medicine and Center for Applied Medical Research (CIMA), Spain

Received August 11, 2013; **Accepted** November 6, 2013; **Published** December 23, 2013

Funding: This study was supported by the Japan Society for the Promotion of Science (JSPS) Postdoctoral Fellowship for Foreign Researchers. This study was also partly supported by the HMT Research Grant for Young Leaders in Metabolomics 2012 from Human Metabolome Technologies Inc. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Co-author Naoto Ishibashi is employed by KOWA Company Ltd. This study was partly supported by the HMT Research Grant for Young Leaders in Metabolomics 2012 from Human Metabolome Technologies Inc. There are no patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

Hepatocellular carcinoma (HCC) represents approximately 85% of all primary liver cancers and is one of the most common malignancies worldwide, especially in Eastern Asia [1]. The prognosis of HCC remains very poor; this poor prognosis is due in part to its high rate of recurrence after initial treatment, which reaches approximately 70% within 5 years [2]. Acyclic retinoid (ACR), a synthetic retinoid with a vitamin A-like structure, prevents the recurrence and development of HCC in patients after the surgical removal of primary tumors [3,4]. ACR is currently undergoing phase II/III clinical trials (JapicCTI-121828) in Japan and is expected to become the first chemopreventive agent.

Another important characteristic of ACR is that it selectively suppresses the growth of HCC cells (JHH7 and others) but not normal hepatic cells (Hc) [5,6]. Although the mechanism underlying this effect is not fully understood, previous basic and clinical studies by our group and others have suggested that both non-genomic and genomic signaling pathways may be responsible for the cancer-selectivity of ACR [5,7,8,9,10,11,12]. A typical example is the prevention by ACR of the aberrant hyper-phosphorylation and inactivation of retinoid X receptor (RXR) α

that occurs during carcinogenesis in HCC [12] and the subsequent induction of apoptosis in HCC cells by the restoration of the expression of RXRα downstream genes such as p21 [11], transglutaminase 2 (TG2) [5] and more. However, to the best of our knowledge, no information is available regarding the effect of ACR on the metabolism of HCC cells.

Recently, the approach of targeting cancer metabolism to develop and improve cancer therapeutics has received a great deal of attention [13]. A distinguishing feature of cancer is that the metabolic pathways of cancer cells are adapted to support rapid and uncontrolled cell proliferation. One of the best-known alterations in cancer cell metabolism is a switch from mitochondrial oxidative phosphorylation to cytoplasmic glycolysis; this switch is known as the Warburg effect [14]. It is possible that targeting cellular metabolism may suppress cancer. In fact, several metabolism-targeting therapies have been already proven to be effective in the treatment of diverse human tumors [13,15].

Although chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infections are believed to account for approximately 80% of HCC [16], a growing body of evidence indicates that metabolic syndrome is also a risk factor for the development of HCC [17]. Indeed, it is extremely difficult to find a single essential target for cancer therapeutics, due to the remarkable heterogeneity and adaptability of cancer cells. It is likely that further investigations into the effect of ACR on cancer cell metabolism will improve our understanding of the molecular pathways underlying the cancer-selective growth suppressive effect of ACR and benefit the development of more effective cancer drugs and therapies against HCC. To achieve this, both nuclear magnetic resonance (NMR)-based and capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS)-based metabolome analyses were performed in JHH7 and Hc cells after treatment with ACR.

Materials and Methods

Materials

ACR (NIK-333) was supplied by Kowa Co. Ltd. (Tokyo, Japan). All-*trans*-retinoic acid (AtRA) was purchased from Sigma-Aldrich (St Louis, MO, USA). Ethanol (EtOH) was obtained from Wako Industries (Osaka, Japan), and used as the primary solvent for all reagents. EtOH solutions were further diluted into cell culture media for treatments. The final concentration of EtOH in media used as a control was 0.05% (vol/vol).

Cell culture

The JHH7 HCC cell line was kindly supplied by Dr. Matsuura (Jikei University School of Medicine, Tokyo, Japan) [18]. The normal human hepatocyte cell line (Hc) was purchased from Cell Systems (Kirkland, WA, USA). Both cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM; Wako Industries) containing 10% fetal bovine serum (FBS, Mediatech, Herndon, VA, USA), 100 U/ml penicillin/streptomycin and 2 mmol/L L-glutamine (Mediatech, Herndon, VA, USA) and grown at 37°C in a humidified 5% CO_2 incubator. For chemical treatment, the cells were cultured in serum-free media containing EtOH or ACR at the appropriate concentrations.

NMR-based metabolomics

For NMR analyses, cells (approximately 1×10^7 cells) treated with EtOH control or 10 μM ACR control for 4 h or 18 h were harvested by scraping as previously described [19]. The one-dimensional (1D) ^{1}H spectra were measured at 500 MHz on a Varian Unity INOVA-500 spectrometer. All NMR spectra were processed using the MestReNova program (Version 5.3.0, MestRec, Santiago de Compostela, Spain). Metabolites were identified using publicly accessible databases, including BioMagRes data bank (http://www.bmrb.wisc.edu), the Metabolomics Database of Linkoping (http://www.mdl.imv.liu.se), and the Human Metabolome Data Bank (http://www.hmdb.ca). Detailed NMR methods have been described previously [19,20].

CE-TOFMS analyses

JHH7 and Hc cells (approximately 5×10^6 cells) treated with EtOH control or 10 μM ACR for 24 h were washed twice with a 5% mannitol solution, and then 1,300 μL of a methanol solution containing 10 μM internal standards was added. Metabolome extraction was then performed as previously described [21]. The metabolic profiles of the cells were then measured using a CE-TOFMS-based metabolomics technique, which is a novel strategy for analyzing and differentially displaying metabolic profiles [21]. CE-TOFMS was carried out using an Agilent CE Capillary Electrophoresis System equipped with an Agilent 6210 Time-of-Flight mass spectrometer, Agilent 1100 isocratic HPLC pump, Agilent G1603A CE-MS adapter kit, and Agilent G1607A CE-ESI-MS sprayer kit (Agilent Technologies, Waldbronn, Germany).

Data analysis for CE-TOFMS and metabolite identification

The raw data obtained by CE-TOFMS were analyzed using KEIO MasterHands software exactly as previously described [22,23]. Briefly, the injected volume for CE and the sensitivity of MS were corrected using internal standards, and then all the annotated metabolites were further corrected to the same chemicals in a standard mixture to overcome different ionization patterns. The peaks were identified based on the matched mass-to-charge ratio (m/z) values and normalized migration times of the corresponding standard compounds.

Real-time RT-PCR

For PCR analyses, RNA was isolated from each cell culture treated with EtOH, AtRA or ACR for 4 h using an RNeasy Kit (Qiagen, Valencia, CA, USA), and the amount and purity of the isolated RNA were evaluated using a NanoDrop spectrophotometer (NanoDrop products, Wilmington, DE, USA). cDNA was then synthesized using a PrimeScript RT Master Mix Kit (TaKaRa Bio, Otsu, Japan). Oligonucleotide primers were designed using OligoPerfect Designer software (Invitrogen, Carlsbad, CA, USA; http://www.tools.invitrogen.com) and synthesized by Invitrogen. The sequences of the primers and the full gene names are summarized in Table S1. PCR reactions were performed using a the Thermal Cycler Dice™ Real Time System (TP8000; Takara Bio) with SsoAdvanced™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA).

Western blot analysis

JHH7 and Hc cells treated with EtOH, AtRA and ACR for 24 h were lysed using RIPA buffer. After boiling at 97°C for 10 min, the protein samples were resolved by sample buffer for sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, run on a 10% gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) and 0.1% Tween and then probed with primary antibodies against pyruvate dehydrogenase kinase 4 (PDK4; sc-14492; 1:1,000 dilution, Santa Cruz Biotechnology, CA, USA), pyruvate dehydrogenase (lipoamide) alpha 1 (PDHA1; sc-377092; 1:1,000 dilution, Santa Cruz Biotechnology), phospho-PDHA1 (ab92696; 1:1,000 dilution, Abcam) or Lamin B1 (ab16048; 1:5,000 dilution, Santa Cruz

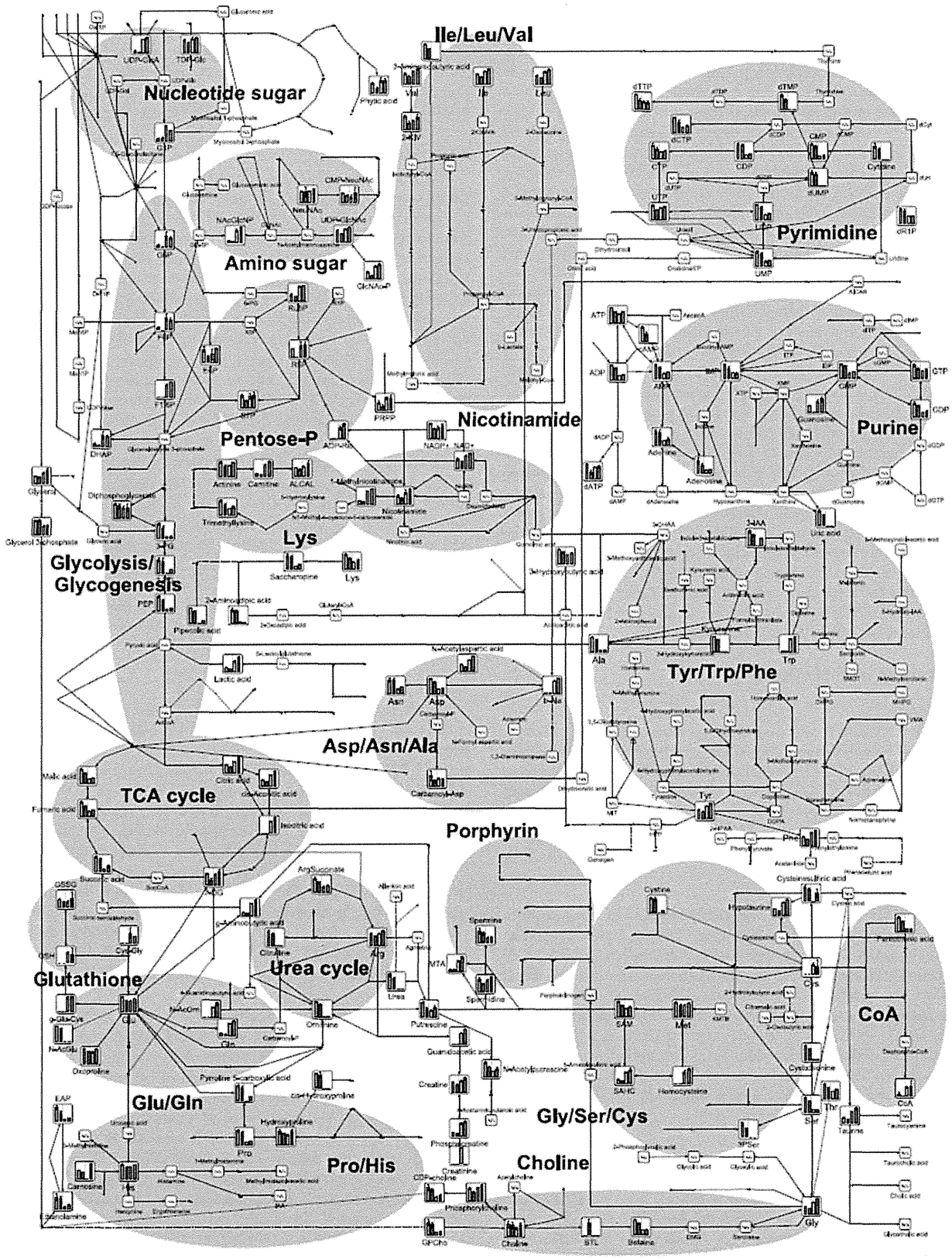

Nucleotide sugar
Ile/Leu/Val
Pyrimidine
Amino sugar
Nicotinamide
Pentose-P
Purine
Lys
Glycolysis/
Glycogenesis
Tyr/Trp/Phe
Asp/Asn/Ala
TCA cycle
Porphyrin
Glutathione
Urea cycle
CoA
Glu/Gln
Gly/Ser/Cys
Pro/His
Choline

Figure 1. Metabolites in the principle metabolic pathways of EtOH- or ACR-treated JHH7 and Hc cells detected by CE-TOFMS. The relative quantities of the detected metabolites are represented as bar graphs (from left to right: EtOH-treated JHH7, ACR-treated JHH7, EtOH-treated Hc, and ACR-treated Hc). N.D., not detected.
doi:10.1371/journal.pone.0082860.g001

Biotechnology). The blots were then incubated with horseradish peroxidase-conjugated anti-goat, anti-mouse or anti-rabbit secondary antibodies and detected using the Amersham ECL PlusTM Western Blotting Detection System (GE Healthcare UK, Buckingham, England). Immunoreactive bands were quantified using ImageJ densitometry software (National Institutes of Health, Bethesda, MD), and normalized; the density of the corresponding band in the EtOH control was set to 1.0.

RNA interference

An siRNA targeting human PDK4 (sc-39030) and a control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology. JHH7 cells were plated in either 96-well plates (1×10^4 cells/well) for cell proliferation analysis and RNA isolation or 60-mm dishes (3.5×10^5 cells/dish) for ATP assays 1 day prior to transfection. The cells were then transfected with 50 nM or 100 nM siRNAs using Lipofectamine 2000 (Life Technologies, Grand Island, NY, USA).

ATP assay

The cellular levels of ATP were measured using a firefly bioluminescence assay kit (AMERIC-ATP kit, Wako Industries) according to the manufacturer's instructions. The luciferase activity was measured using a plate reader (ARVO MX, Perkin Elmer Inc., MA, USA).

Cell viability assay

The number of viable cells was determined using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Tokyo, Japan) as previously described [5].

Network generation and pathway analyses

The Ingenuity Pathways Analysis (IPA) program (Ingenuity Systems, Mountain View, CA, USA; http://www.ingenuity.com) was used to identify networks and canonical pathways as previously described [24]. The generated biological networks were ranked by score, which is the likelihood that a set of genes is found in the networks due to random chance as measured by a Fisher's exact test. The resulting canonical pathways were ranked by *P* values, which were calculated using a Fisher's exact test by comparing the number of user-specified genes of interest that participate in a given function or pathway, relative to the total number of occurrences of these genes in all the functional/pathway annotations stored in the Ingenuity Pathways Knowledge Base [25].

GEO data mining

The normalized PDK4 expression from a clinical data set, which contains transcriptome profiling of 268 HCC tumor, 243 adjacent non-tumor, 40 cirrhotic and 6 healthy liver samples, was downloaded from the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo, accession no. GSE25097).

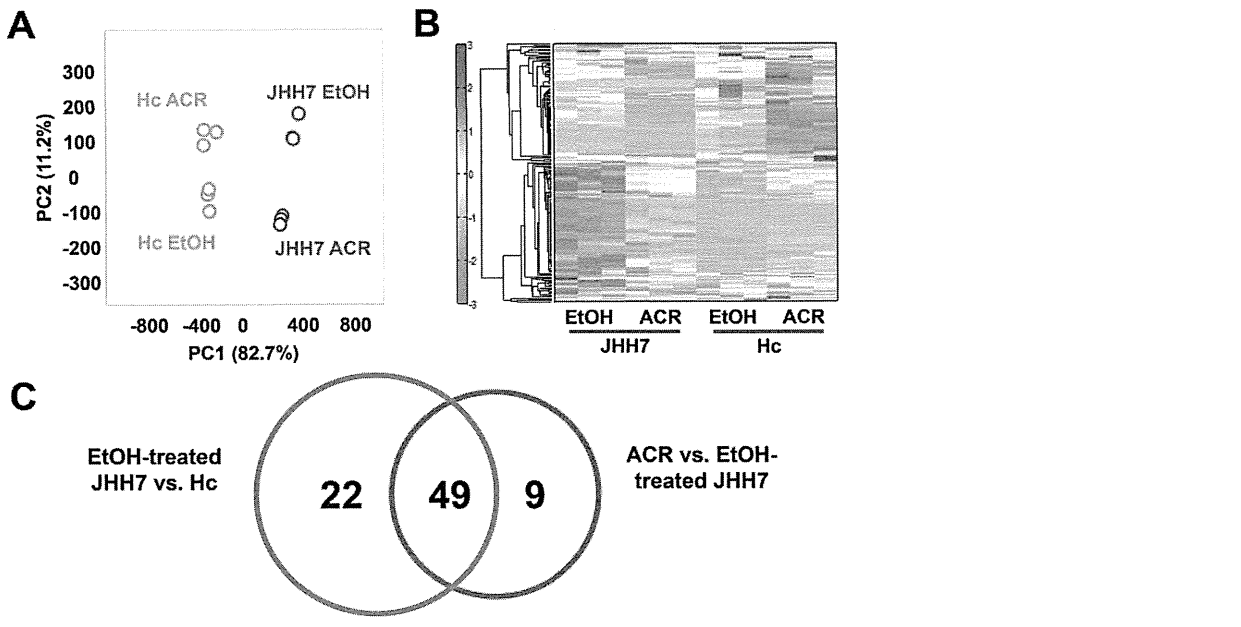
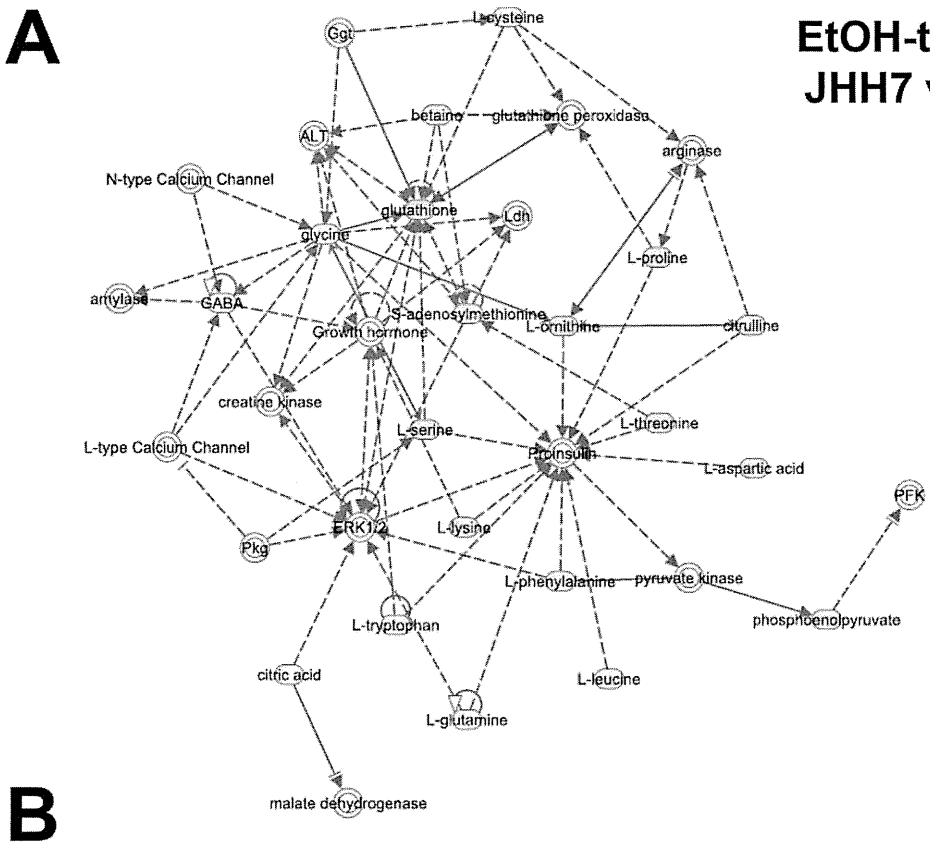


Figure 2. A comparison of the metabolic profile of EtOH- or ACR-treated JHH7 and Hc cells determined by CE-TOFMS. PCA scoreplot (*A*) and heat map (*B*) from metabolic data of JHH7 and Hc cells treated with EtOH and ACR ($n=3$). Venn-diagrams (*C*) showing the number of metabolites that were significantly deregulated between the two groups.
doi:10.1371/journal.pone.0082860.g002


A
EtOH-treated JHH7 vs. Hc
L-cysteine
Ggt
betaine
glutathione peroxidase
ALT
arginase
N-type Calcium Channel
glutathione
Ldh
glycine
L-proline
amylase
GABA
S-adenosylmethionine
L-ornithine
citrulline
Growth hormone
creatine kinase
L-serine
L-threonine
L-type Calcium Channel
Proinsulin
L-aspartic acid
PFK
ERK1/2
L-lysine
Pkg
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pyruvate kinase
L-tryptophan
phosphoenolpyruvate
citric acid
L-leucine
L-glutamine
malate dehydrogenase

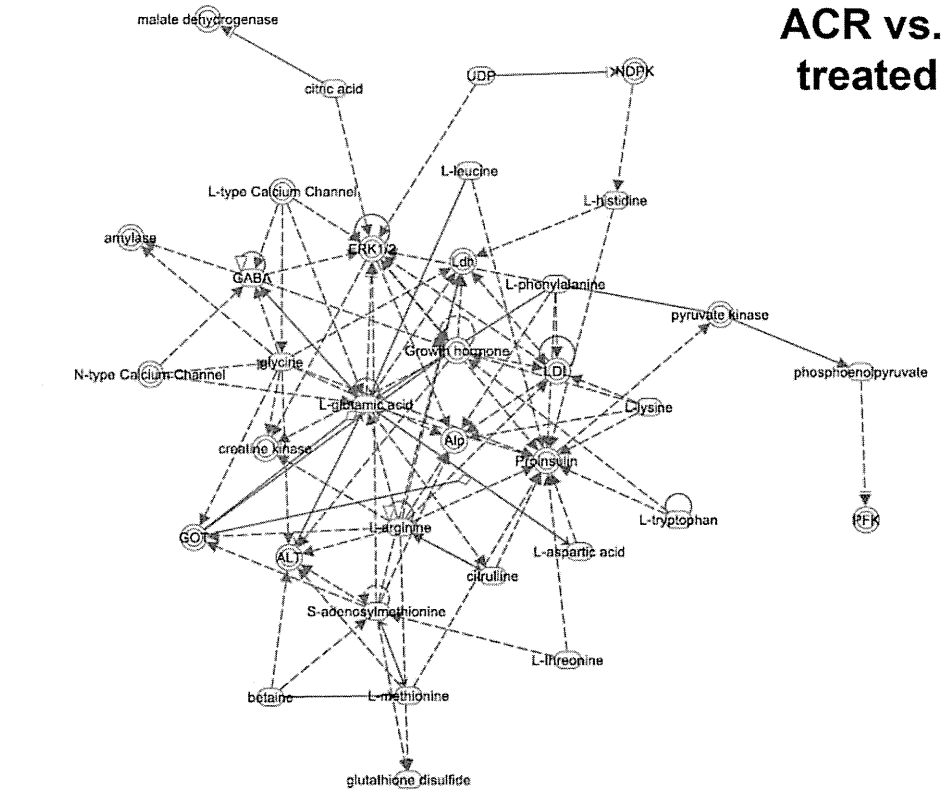
B
ACR vs. EtOH-treated JHH7
malate dehydrogenase
citric acid
UDP
NDPK
L-leucine
L-type Calcium Channel
L-histidine
amylase
ERK1/2
Ldh
GABA
L-phenylalanine
pyruvate kinase
Growth hormone
glycine
LDL
N-type Calcium Channel
phosphoenolpyruvate
L-glutamic acid
L-lysine
Alp
creatine kinase
Proinsulin
L-tryptophan
PFK
GOT
L-arginine
L-aspartic acid
ALT
citrulline
S-adenosylmethionine
L-threonine
betaine
L-methionine
glutathione disulfide

Figure 3. Network generation using Ingenuity Pathway Analysis. The "Increased Levels of Albumin, Amino Acid Metabolism, Molecular Transport" network was associated with metabolites that were significantly different between JHH7 and Hc cells (*A*) and the metabolites that were differentially regulated by ACR in JHH7 cells (*B*). Up-regulated metabolites are indicated in red, down-regulated metabolites indicated in green, and metabolites that were not annotated in this study but are part of this network are indicated in white. Direct relationships are drawn with solid arrows, and indirect relationships are drawn with dashed arrows.
doi:10.1371/journal.pone.0082860.g003

Statistical and multivariate analyses

All the experiments in this study were performed independently two or more times to ensure the reproducibility of the results. Quantitative data were expressed as the means ± SEMs. The statistical significance of differences between values was assessed using a two-tailed Student's *t*-test or a Mann-Whitney U test. Values of $P<0.05$ were considered to indicate statistical significance. Unsupervised principal component analysis (PCA) was run using SIMCA-P+ software (Version 12.0, Umetrics, Umeå, Sweden).

Results

The effect of ACR on the metabolism of JHH7 cells detected using ^{1}H-NMR

First, NMR-based metabolomics was performed to investigate the effect of ACR treatment on the metabolism of JHH7 cells. As shown in Figure S1, PCA analysis of the NMR spectra indicated that treatment with ACR for 4 h had a very minor effect on the metabolism of JHH7 cells, while obvious changes were observed after 18 h of ACR treatment compared to the EtOH control.

Differences between the metabolic profiles of JHH7 and Hc cells treated with EtOH and ACR detected using CE-TOFMS

To further investigate the cancer-selective effect of ACR, the metabolic profiles of JHH7 and Hc cells treated with EtOH and ACR for 24 h was measured using CE-TOFMS analysis. A total of 229 peaks (109 cationic and 120 anionic) were detected in either JHH7 or Hc cells; from these 229 peaks, 88 principal metabolites were quantified (Table S2). The metabolic pathways of all the detected metabolites are illustrated in Figure 1. These metabolites are associated with glycolysis/gluconegenesis, the pentose phosphate pathway, the tricarboxylic acid cycle, the urea cycle, pyrimidine metabolism, nicotinate and nicotinamide metabolism and amino acid metabolism. The result of the comparison of the metabolic profiles of the cells is provided in Figure 2. PCA analysis revealed a very clear distinction between the abundance of intracellular metabolites of JHH7 and Hc cells with and without ACR treatment (Figure 2A), while the first component (PC1) indicated that 67% of the total variance is due to the difference between JHH7 and Hc cells. PC2 (11.2%) indicated that the ACR-treated JHH7 cells have a metabolic profile that is similar to that of the EtOH-treated Hc cells. Furthermore, heatmap analyasis indicated that the metabolic pattern of JHH7 cells was almost completely opposite that of the Hc cells; a similar difference was observed between the ACR-treated and EtOH-treated JHH7 cells (Figure 2B). Finally, the cellular content of 71 metabolites in JHH7 and Hc cells was significantly different with *P* values less than 0.05 and fold changes greater than 1.2; 58 metabolites were significantly down-regulated by ACR in JHH7 cells compared to the EtOH control. Forty-nine common metabolites were shared between the two groups (Figure 2C).

Network generation and pathway analyses

Next, the list of the significantly different metabolites was imported into the IPA platform to investigate possible biological interactions. The biological functions of the top five IPA-generated networks and top five canonical metabolic pathways are summarized in Tables 1 and 2, respectively, and shown in Figure 3. Interestingly, IPA analysis indicated that the most highly populated biological network ("Increased Levels of Albumin, Amino Acid Metabolism, Molecular Transport") and the top two canonical metabolic pathways ("tRNA Charging" and "Purine Nucleotides De Novo Biosynthesis II") that were associated with the ACR-regulated metabolites by in JHH7 cells were the same as the networks that were associated with metabolic differences between JHH7 and Hc cells.

Table 1. Top five associated network functions generated by IPA.

	Top function	Score
EtOH-treated JHH7 vs. Hc	Increased Levels of Albumin, Amino Acid Metabolism, Molecular Transport	39
	Cellular Growth and Proliferation, Organismal Development, Cellular Compromise	21
	Cardiovascular System Development and Function, Organ Development, Carbohydrate Metabolism	18
	Cellular Growth and Proliferation, Organismal Development, Small Molecule Biochemistry	16
	Carbohydrate Metabolism, Cell Morphology, Cell-To-Cell Signaling and Interaction	11
ACR vs. EtOH-treated JHH7	Increased Levels of Albumin, Amino Acid Metabolism, Molecular Transport	38
	Carbohydrate Metabolism, Molecular Transport, Small Molecule Biochemistry	23
	Cellular Growth and Proliferation, Organismal Development, Small Molecule Biochemistry	16
	Free Radical Scavenging, Small Molecule Biochemistry, Molecular Transport	14
	Post-Translational Modification, Cellular Assembly and Organization, Developmental Disorder	6

doi:10.1371/journal.pone.0082860.t001

Table 2. Top canonical pathways identified by IPA.

	Top canonical pathway	P-Value
EtOH-treated JHH7 vs. Hc	tRNA Charging	6.82E-30
	Purine Nucleotides De Novo Biosynthesis II	1.30E-24
	Pyrimidine Ribonucleotides De Novo Biosynthesis	9.94E-20
	Superpathway of Citrulline Metabolism	9.93E-19
	Gluconeogenesis I	8.21E-18
ACR vs. EtOH-treated JHH7	tRNA Charging	9.48E-30
	Purine Nucleotides De Novo Biosynthesis II	2.91E-19
	Arginine Biosynthesis IV	3.10E-15
	Citrulline-Nitric Oxide Cycle	1.84E-14
	NAD biosynthesis II (from tryptophan)	5.72E-14

doi:10.1371/journal.pone.0082860.t002

ACR inhibits the increase in adenosine-5′-triphosphate (ATP) production in JHH7 cells

A comparison of the biosynthetic metabolites (nucleotides, amino acids and lipids) in the EtOH- or ACR-treated JHH7 and Hc cells determined by CE-TOFMS is summarized in Table 3. Of particular interest, the changes in the concentrations of adenosine nucleotides are shown in Figure 4. Notably, ATP levels were 1.6-fold higher in the EtOH-treated JHH7 cells than in the EtOH-treated Hc cells; ACR suppressed this increase, nearly to the basal levels observed in Hc cells (0.72-fold and $P = 0.00015$ compared to the EtOH-treated JHH7 cells). In contrast, only a very minor effect of ACR was observed on the levels of adenosine diphosphate (ADP) and adenosine monophosphate (AMP) in JHH7 cells (0.84- and 0.82-fold compared to the EtOH control, respectively).

ACR enhances PDK4 expression in JHH7 cells, but not in Hc cells

To further understand the cancer-selective inhibitory effect of ACR on ATP production, a set of genes that is known to be important in the regulation of energy metabolism in cancer cells was selected based on previous reports [26,27,28,29], and the effect of ACR on the expression of these genes was measured using real-time PCR (Figure 5A). Of particular interest, we found that ACR significantly enhanced the expression of PDK4, an important regulator of ATP levels [30], in JHH7 cells but not in Hc cells (3.06-fold; $P = 0.0033$ and 1.20-fold; $P = 0.062$, respectively; Figure 5B). Further western blot analysis revealed a nearly 2-fold increase in PDK4 protein levels after ACR treatment, but ACR did not affect the phosphorylation of PDHA1 in JHH7 cells (Figure 5C).

Functional analysis of PDK4 in JHH7 cells

Furthermore, loss-of-function experiments were performed to confirm the role of PDK4 in the effect of ACR on cellular ATP levels and the proliferation of JHH7 cells. As shown in Figure 6A, treatment with an siRNA targeting PDK4 (siPDK4) caused a dose-dependent downregulation of PDK4 mRNA expression (0.57-fold and 0.41-fold compared to siControl-treated cells with 50 nM and 100 nM siPDK4, respectively). Interestingly, ACR weakly but significantly inhibited cellular ATP levels in siControl-treated JHH7 cells (0.88-fold and $P = 0.042$ compared with EtOH). In contrast, no significant effect was observed in siPDK4-treated JHH7 cells (1.07-fold and $P = 0.42$ compared with EtOH; Figure 6B). However, PDK4 knockdown did not rescue the inhibitory effect of ACR on the proliferation of JHH7 cells (Figure 6C).

Clinical expression levels of PDK4

The mining of microarray data from a human HCC data set revealed that PDK4 mRNA is significantly down-regulated in liver tumors compared to adjacent non-tumor liver tissues (0.66-fold, $P = 3.11E\text{-}85$; Figure 7A). Finally, a PDK4-dependent regulatory network that involves RXR and peroxisome proliferator-activated

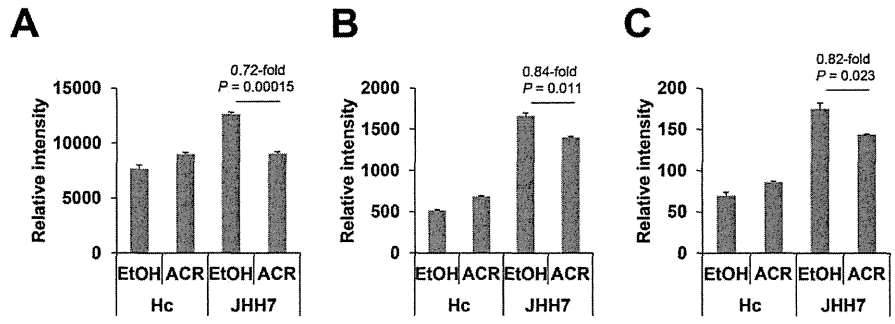

Figure 4. Levels of adenosine nucleotides in EtOH or ACR-treated JHH7 and Hc cells determined by CE-TOFMS. ATP (*A*), ADP (*B*) and AMP (*C*) levels.
doi:10.1371/journal.pone.0082860.g004