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REVIEW

Pharmaceutical and nutraceutical approaches for preventing liver carcinogenesis: Chemoprevention of hepatocellular carcinoma using acyclic retinoid and branched-chain amino acids

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The poor prognosis for patients with hepatocellular carcinoma (HCC) is associated with its high rate of recurrence in the cirrhotic liver. Therefore, more effective strategies need to be urgently developed for the chemoprevention of this malignancy. The malfunction of retinoid X receptor α, a retinoid receptor, due to phosphorylation by Ras/mitogen-activated protein kinase is closely associated with liver carcinogenesis and may be a promising target for HCC chemoprevention. Acyclic retinoid (ACR), a synthetic retinoid, can prevent HCC development by inhibiting retinoid X receptor α phosphorylation and improve the prognosis for this malignancy. Supplementation with branched-chain amino acids (BCAA), which are used to improve protein malnutrition in patients with liver cirrhosis, can also reduce the risk of HCC in obese cirrhotic patients. In experimental studies, both ACR and BCAA exert suppressive effects on HCC development and the growth of HCC cells. In particular, combined treatment with ACR and BCAA cooperatively inhibits the growth of HCC cells. Furthermore, ACR and BCAA inhibit liver tumorigenesis associated with obesity and diabetes, both of which are critical risk factors for HCC development. These findings suggest that pharmaceutical and nutraceutical approaches using ACR and BCAA may be promising strategies for preventing HCC and improving the prognosis of this malignancy.

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1 Introduction

Hepatocellular carcinoma (HCC), which usually develops in the livers of patients with chronic hepatitis and liver cirrhosis, is a serious clinical and social issue worldwide. Annually, the number of new cases is approximately 750 000, with an estimated 700 000 patients dying because of the malignancy [1, 2]. Although effective methods of diagnosis and treatment for HCC have been recently developed, improvement in the prognosis for this cancer is limited; overall survival, 10 years after curative treatment, is only 22–35% [3, 4]. The primary reason for the poor prognosis of HCC is its high frequency of recurrence after curative treatment; the recurrence rate, 5 years after definitive therapy in cirrhotic patients, may exceed 70% [5–7]. These facts indicate that curative treatment for HCC is difficult once this malignancy has developed, and therefore, effective strategies for preventing this cancer are urgently required.

In a previous, prospective, randomized trial, we reported that the oral administration of acyclic retinoid (ACR), a novel synthetic retinoid, significantly suppressed the posttherapeutic recurrence of HCC and improved the survival rate of patients [8–10]. Oral supplementation with branched-chain

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Abbreviations: ACR, acyclic retinoid; **BCAA**, branched-chain amino acids; **ERK**, extracellular signal-regulated kinase; **HBV**, hepatitis B virus; **HCC**, hepatocellular carcinoma; **HCV**, hepatitis C virus; **IGF**, insulin-like growth factor; **IGF-1R**, IGF-1 receptor; **MAPK**, mitogen-activated protein kinase; **PEM**, protein energy malnutrition; **PI3K**, phosphoinositide-3-kinase; **RAR**, retinoic acid receptor; **RTK**, receptor tyrosine kinase; **RXR**, retinoid X receptor

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amino acids (BCAA), which is widely used in patients with liver cirrhosis to improve protein energy malnutrition (PEM), also reduced the risk of HCC in obese cirrhotic patients [11]. The effects of ACR and BCAA on the chemoprevention of HCC and the inhibition of HCC cell growth have been reported in several experimental studies [12–16]. In particular, recent rodent studies demonstrated that administration of ACR and BCAA suppresses the liver carcinogenesis associated with obesity and diabetes, both of which are critical risk factors for HCC development [17, 18]. The results of these clinical and basic studies strongly suggest that pharmaceutical and nutraceutical approaches, especially using ACR and BCAA, might be effective strategies for preventing liver carcinogenesis. In this article, we provide an overview of the clinical characteristics and molecular pathogenesis of HCC, focusing on the role of retinoid X receptor α (RXRα) phosphorylation in liver carcinogenesis. The detailed effects of ACR and BCAA in the prevention of HCC development are reviewed, based on our clinical and basic research. We also review the possibility of pharmaceutical and nutraceutical approaches for the inhibition of obesity- and diabetes-related liver carcinogenesis through the targeting of the pathophysiological conditions caused by these metabolic abnormalities, concentrating on the effects of ACR and BCAA.

2 Clinical characteristics of HCC

Most cases of HCC, which is the dominant form of primary liver carcinoma, are associated with the chronic inflammation and subsequent cirrhosis of the liver, that is induced by a persistent infection with one of the hepatitis viruses, hepatitis B virus (HBV) or hepatitis C virus (HCV) [19, 20]. After development of virus-induced chronic hepatitis and liver cirrhosis, the entire liver enters a precancerous state, possessing multiple, independent, premalignant, or latent malignant clones. Therefore, the typical clinical pattern of liver carcinogenesis is **ulticentric carcinogenesis, which is also described as **ield cancerization. This carcinogenesis pattern contributes to the high frequency of HCC development in patients with viral liver cirrhosis. Significantly, the annual rate for HCC development is approximately 7% in cirrhotic patients, and even after curative treatment, the annual incidence of recurrence is approximately 20–25% [5–7]. These facts highlight the poor prognosis of viral liver cirrhotic patients and suggest the possibility of improved clinical outcomes if effective strategies are developed for preventing HCC.

One of the most effective approaches for preventing the development of HCC is the eradication of the hepatitis viruses. Several meta-analyses have shown the effectiveness of IFN therapy for preventing HCV-related HCC [21–23], indicating that sustained antiviral response to IFN-based therapy is associated with a reduced risk of developing this malignancy. In addition, IFN treatment might be effective for preventing HCC development in HCV patients, even if sustained antiviral response is not achieved [24]. Antiviral treatments, such as IFN therapy and nucleos(t)ide analog therapy, also prevent the development of HBV-related HCC [25, 26]. These clinical evidences strongly suggest that antiviral treatment is effective for reducing the incidence of HCC development in patients with chronic HBV or HCV infections. In addition, two cohort studies of HCV patients demonstrated that hepatic inflammation alleviation therapy, involving glycyrrhizin injection, suppressed HCC development [27, 28]. These results also indicate that attenuation of chronic inflammation might be effective for inhibiting liver carcinogenesis.

3 Molecular pathogenesis of HCC

HCC is a heterogeneous tumor because it develops in a complex multistep process in which many signaling cascades are altered. That is, the accumulation of genetic alterations is critically involved in hepatocarcinogenesis [29, 30]. Genomic mutations in the *p53* tumor suppressor gene occur in 10–35% of HCC cases [31]. Genomic mutations in the *CTNNB1* gene, which encodes β-catenin, have also identified in approximately 20–40% of liver cancers [31]. Because of these alterations, several signaling pathways related to cell proliferation and survival are activated during liver carcinogenesis. For instance, epithelial growth factor receptor, which is a receptor tyrosine kinase (RTK), is expressed in 68% of HCC cases, and this receptor is associated with the proliferation and clinical stage of this malignancy [32]. Activation of insulin-like growth factor (IGF) 1 receptor (IGF-1R) signaling, which is another RTK, also contributes to the early stages of liver carcinogenesis [30]. The major signaling pathways activated by the RTKs/Ras pathways are the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and the phosphoinositide-3-kinase (PI3K)/Akt pathways, both of which play important roles in the proliferation and survival of cancer cells. These reports, therefore, strongly suggest that targeting specific RTKs and their downstream signaling pathways is a potentially effective strategy for preventing some types of human malignancies, including HCC [33–38].

4 Retinoid abnormalities and HCC

In addition to the pathophysiological mechanisms as mentioned above, recent studies have revealed the magnitude of the abnormalities in the expression and function of retinoids on liver carcinogenesis [12–15, 39]. Retinoids are a group of natural and synthetic molecules that are structurally and/or functionally related to fat-soluble vitamin A. These molecules participate in a broad spectrum of biological activities, including embryogenesis, growth, differentiation, proliferation, apoptosis, and metabolism [40–42]. The fundamental effects of retinoids on cellular activities are largely mediated through the expression of two distinct families of nuclear receptors, the retinoic acid receptors (RARs) and RXRs. The RARs are activated by all-*trans*-retinoic acid and 9-*cis*-retinoic

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acid, with similar affinities, whereas RXRs are only activated by 9-*cis*-retinoic acid [40–42]. Both the RARs and RXRs are composed of three subtypes (α, β, and γ), which are characterized by a modular domain structure, and these nuclear receptors are ligand-dependent transcription factors [40–42]. After ligand binding, the RXRs form homodimers and heterodimers with the RARs and interact with the retinoid X response element or the RAR responsive element, which are located in the promoter region of the target genes, thereby modulating gene expression [40–42]. RXRs can also form heterodimers with other nuclear receptors, such as peroxisome proliferator-activated receptor, indicating that RXRs act as common heterodimerization partners for various types of nuclear receptors [41]. Thus, RXRs are considered the master regulators of nuclear receptors because they are involved in the regulation of fundamental cell activities, including normal cell proliferation, metabolism, and death (regulation of apoptosis). In particular, RXRα plays a critical role in the normal control of hepatocyte lifespan and proliferation [43, 44].

These characteristics also suggest that abnormalities in the expression and function of retinoid signaling are closely associated with deviations from normal cell proliferation and death, which are key factors in the development of several types of human cancers, including HCC. For example, retinol, a transport form of retinoid in the plasma, is locally deficient in HCC, but not in the adjacent, normal liver tissue in a rodent model of hepatocarcinogenesis [45]. In a rat model of chemically induced liver carcinogenesis, repression of RXRα occurs even in the early stages of carcinogenesis because its expression is decreased not only in HCC and liver cell adenoma, but also in precancerous HCC lesions [46]. The expression levels of RARβ, which is regarded as a tumor suppressor gene because of its ability to regulate cell growth and apoptosis [47], are markedly decreased in both human [48] and rat HCC [46]. On the other hand, RARγ, which is overexpressed in human HCC tissues and cells, enhances the growth of HCC cells through the activation of the PI3K/Akt signaling pathway [49]. These reports strongly indicate that the restoration of the function and expression of retinoid receptors, via treatment with retinoids, might be effective for the prevention of certain types of human malignancies, including HCC [12–15, 50, 51].

5 RXRα phosphorylation and HCC

We proposed that RXRα phosphorylation and its malfunction is closely associated with liver carcinogenesis [12–15]. RXRα protein, which is anomalously phosphorylated at its serine and threonine residues, prominently accumulates in both surgically resected human HCC tissues and human HCC-derived cell lines [39, 52]. Activation of the RTK/Ras/MAPK signaling frequently occurs in HCC cells [30, 32]. The constitutive phosphorylation of serine-260 in RXRα, a MAPK/ERK consensus site, by this signaling pathway is closely associated with retarded degradation of RXRα, lowered transcriptional activity of this nuclear receptor, and promotion of cancer cell growth [39, 53]. In human HCC cells, phosphorylated RXRα is resistant to proteolytic degradation via the ubiquitination-/proteasome-mediated pathway, facilitating the accumulation of this phosphorylated protein within HCC tissues [54]. Furthermore, phosphorylated RXRα abolishes its ability to form heterodimers with RARβ, and this is implicated in uncontrolled cell growth and retinoid resistance [55]. These findings suggest that the accumulation of phosphorylated RXRα, regarded as the nonfunctional form of RXRα, may interfere with the function of normal (unphosphorylated) RXRα in a dominant-negative manner, thus, playing a critical role in liver carcinogenesis (Fig. 1). On the other hand, the abrogation of RXRα phosphorylation by a MAPK inhibitor or transfection with the nonphosphomimetic mutant RXRα restores the degradation of RXRα in a ligand-dependent manner [39, 53]. Thus, the targeting of RXRα phosphorylation might be a strategy for preventing HCC, and ACR is a promising agent for this purpose, as discussed in Section 6.

6 Mechanisms of ACR in HCC chemoprevention

ACR, also known as NIK-333 and Peretinoin (Kowa Pharmaceutical, Tokyo, Japan), is a synthetic retinoid that was initially developed as an agonist for both RXR and RAR [56, 57]. ACR inhibits growth of human HCC-derived cells by activating the promoter activity of retinoid X response element and RAR responsive element and regulating the expression of retinoid target genes, including *RARβ*, *p21^{CIP1}*, and *cyclin D1*, resulting in the induction of apoptosis and cell cycle arrest in the G_0–G_1 phase [53, 58–63]. These findings indicate that ACR exerts growth inhibitory effects in HCC cells, at least in part, by working as a ligand for retinoid receptors and controlling their target genes, especially *RARβ* and *p21^{CIP1}*. The antitumor effects of ACR are also associated with suppression of telomerase activity, attenuation of oxidative stress, and inhibition of angiogenesis [64–66]. Moreover, the suppressive effects of ACR on liver carcinogenesis have been demonstrated in several animal experiments [17, 45, 67–69].

Furthermore, we have proposed that inhibition of RXRα phosphorylation is a critical mechanism of ACR, allowing it to exert chemopreventive effects in liver carcinogenesis. In human HCC-derived cells, ACR can restore RXRα function by inactivating the Ras/MAPK signaling system and dephosphorylating RXRα, although 9-*cis*-retinoic acid is incapable of suppressing ERK and RXRα phosphorylation [53]. Moreover, recent studies have revealed that ACR suppresses the growth of several types of cancer cells, such as HCC and head and neck squamous cell carcinoma cells, and prevents chemically induced liver carcinogenesis by inhibiting the activation and expression of several types of growth factors and their corresponding RTKs [63, 66, 68–73]. ACR also inhibits Ras activation, and this is associated with prevention of obesity-related liver tumorigenesis in mice and the

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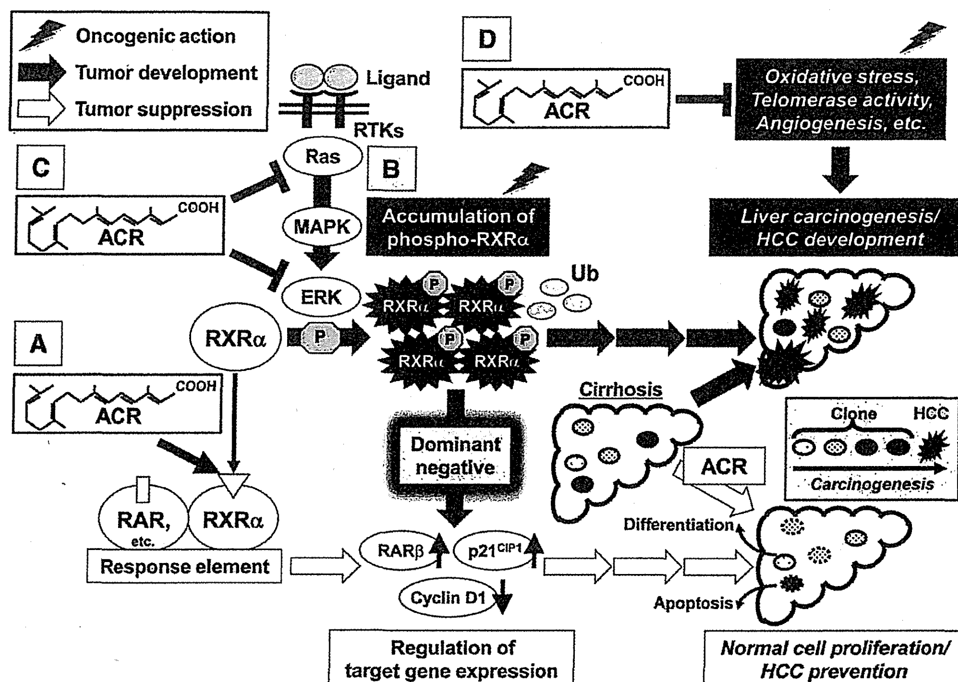

Figure 1. Retinoid refractoriness due to phosphorylation of RXRα, and its restoration by ACR in liver carcinogenesis. When ACR binds to and activates RXRα in normal hepatocytes, the receptor forms homodimers and/or heterodimers with other nuclear receptors, including RARs. This results in the expression of the target genes, such as *RARβ*, *p21*^{CIP1}, and *cyclin D1*, which regulate normal cell proliferation and differentiation and control the induction of apoptosis and cell cycle progression. Therefore, in the cirrhotic liver, ACR can delete and inhibit malignant clones, at least in part, by controlling the expression of these RXRα-target genes (A). In HCC cells, several types of RTKs, such as epidermal growth factor receptor superfamily and IGF-1R and their downstream Ras/MAPK pathway, are highly activated, which results in the phosphorylation of ERK and RXRα and subsequent suppression of dimer formation and transactivation functions of RXRα (refractoriness to retinoid). Furthermore, nonfunctional phosphorylated RXRα, which is sequestered from ubiquitin (Ub)/proteasome-mediated degradation and accumulates in liver cells, interferes with the physiological functions of the remaining nonphosphorylated (i.e., functional) RXRα in a dominant-negative manner, and this is also involved in liver carcinogenesis (B). ACR inhibits phosphorylation of RXRα, restores the function of this receptor, and activates the transcriptional activity of the responsive element associated with this receptor. This is accomplished by inhibiting the Ras/MAPK signaling pathway and the ligand-dependent (growth factor) RTK activities, which contribute to the prevention of liver carcinogenesis and suppression of growth in HCC cells (C). In addition, ACR inhibits growth of HCC cells through the attenuation of oxidative stress, inhibition of telomerase activity, and repression of angiogenesis (D). The pleiotropic effects of ACR to prevent HCC development have also been summarized in recent reviews [12–15].

inhibition of cell growth in human HCC and pancreatic cancer cells [17, 58, 74]. These findings indicate that activation of the RTK/Ras/MAPK signaling pathway, which is involved in HCC development [30, 32], and the subsequent phosphorylation of RXRα are critical targets of ACR for the inhibition of liver carcinogenesis [12–15] (Fig. 1).

7 HCC chemoprevention by ACR: Clinical trial results

Because the results from numerous preclinical experiments indicated that ACR may be an effective agent for HCC chemoprevention, an early-phase, randomized, controlled clinical trial was conducted to determine whether ACR can reduce the incidence and recurrence of second primary HCC in patients who underwent potentially curative treatment for initial HCC [8–10]. In this trial, oral administration of ACR (44 patients, 600 mg/day) for 12 months significantly reduced the incidence of recurrent or new HCC compared to placebo (45 patients) after a median follow-up period of 38 months; 12 patients (27%) in the ACR group developed HCC as compared with 22 patients (49%) in the placebo group ($p = 0.04$) [8]. After a further follow-up period of 62 months, ACR treatment demonstrated improved recurrence-free survival ($p = 0.002$) and overall survival ($p = 0.04$) [9]. The relative risk for the development of secondary HCC and death were 0.31 (95% confidence interval [CI], 0.12–0.78) and 0.33 (95% CI, 0.11–0.79), respectively [8, 9]. Therefore, the estimated 6-year overall survival was 74% in the ACR group and 46% in the placebo group [9].

A multicenter, large-scale ($n = 401$), randomized, placebo-controlled trial also confirmed the effectiveness of ACR in preventing second primary HCCs in HCV-positive patients

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who underwent curative treatment for primary or the first recurrence of HCC, with a median follow-up of 2.5 years. In this trial, oral administration of ACR (600 mg/day) had a strong effect on the prevention of a second primary HCC with a hazard ratio of 0.27 (95% CI, 0.07–0.96), 2 years after treatment, and at 3 years, the cumulative recurrence-free survival rates in the ACR-treated group (43.7%) were higher than those in the placebo group (29.3%) [75]. In addition, a subgroup analysis of this study showed that ACR prevented development of a second primary HCC with a hazard ratio of 0.38 (95% CI, 0.20–0.71) in patients who were Child-Pugh A and had small tumors (size, <20 mm) [76]. These results indicated that ACR administration at an early stage of liver cirrhosis contributes to the prevention of HCC. In addition to the effectiveness of ACR for the prevention of HCC development, the results of these clinical trials [8–10, 75, 76], together with a phase I pharmacokinetics trial [77], have proven the safety of ACR in a clinical setting. Therefore, the findings of these clinical trials [8–10, 75–77] strongly suggest that ACR is a novel first-line therapy for reducing the development of a second primary HCC.

8 HCC chemoprevention by ACR: The concept of "clonal deletion" therapy

Two interesting facts were revealed in an early-phase, ACR clinical trial [8–10]. First, the preventive effects of ACR on HCC development lasted up to 50 months after randomization or 38 months after completion of ACR administration, indicating that a 12-month administration of this agent conferred a long-term effect on the prevention of second primary HCCs [10]. Second, ACR administration for 12 months significantly reduced the serum levels of lectin-reactive α-fetoprotein factor 3, which might be produced from latent (i.e., invisible) malignant clones in the remnant liver [78]. These facts suggest the following two possibilities: (i) ACR can delete the α-fetoprotein factor 3 producing premalignant clones from the remnant liver before they expand into clinically detectable HCC and (ii) after the elimination of the malignant clones from the remnant liver by ACR, several years elapse before the clinical appearance of the next HCC clones. The cirrhotic liver is a precancerous field that possesses multiple, independent premalignant, or latent malignant clones. Therefore, before expanding into clinically detectable tumors, a positive approach for the removal and inhibition of such latent malignant clones from the cirrhotic liver should be conducted to prevent HCC development. We consider that implementation of this approach, termed clonal deletion therapy, is a practical approach for preventing HCC, and that ACR is a consistent and reasonable agent for this purpose [12–15] (Fig. 1).

A recent study by Honda et al. [79] reported that an 8-wk administration of ACR significantly elevated the expression levels of many retinoid target genes and tumor suppressor-related genes, but decreased the expression levels of tumor progression-related genes in the liver of HCV-positive patients. This report may also provide evidence that ACR can change the hepatic environment to a non-hypercarcinogenic one.

9 BCAA supplementation and chronic liver disease

BCAA (valine, leucine, and isoleucine) is a widely accepted therapy for improving hepatic insufficiency and its related PEM, which is a common manifestation of patients with liver cirrhosis [80, 81]. PEM affects the outcome of the cirrhotic patients by determining both their quality of life and survival [82, 83]. Cirrhotic patients frequently demonstrate a decreased serum ratio of BCAA to aromatic amino acids, reduced serum albumin levels, and decreased skeletal muscle volume [80, 81]. They have also demonstrated that an increased consumption of foods containing high BCAA content does not affect plasma BCAA levels [84]. On the other hand, nutritional intervention with BCAA has been shown to increase the serum albumin concentration and improve patient quality of life and prognosis by preventing severe complications associated with this disease [85–88]. For instance, in a multicenter, large scale ($n = 646$), randomized, and nutrient intake-controlled trial in Japan, the long-term survival study, oral supplementation with BCAA (12 g/day) for 2 years to patients with decompensated cirrhosis significantly decreased the incidence of events associated with progression to hepatic failure (hazard ratio, 0.67; 95% CI, 0.49–0.93; $p = 0.015$; median observation period, 445 days) [85]. The reports of the trial [85–88], therefore, indicated that BCAA supplementation may serve as a first-line therapy for patients with decompensated cirrhosis.

10 HCC chemoprevention by BCAA supplementation

Several experimental studies have revealed the precise mechanisms of BCAA in the suppression of cancer cell growth and chemoprevention of HCC. Hagiwara et al. [89] reported that BCAA directly suppresses HCC cell proliferation by inducing apoptosis and inhibiting the activation of PI3K/Akt and nuclear factor-κB signaling pathways. BCAA treatment also inhibits the proliferation of human HCC-derived cells by increasing cellular levels of $p21^{CIP1}$ and arresting the cell cycle in the G_0/G_1 phase [90]. Both in vitro and in vivo studies have demonstrated the antiangiogenesis activity of BCAA induced by suppressing the expression of vascular endothelial growth factor in HCC cell lines and in the liver of rats bearing neoplasm [91, 92]. BCAA supplementation also reduces oxidative stress in HCV-positive patients with liver cirrhosis as well as in rats with advanced liver cirrhosis [93, 94]. These reports suggest that BCAA exerts chemopreventive effects against HCC, at least in part, by suppressing angiogenesis and

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improving oxidative stress, both of which are critically involved in liver carcinogenesis.

Moreover, recent clinical trials revealed that BCAA supplementation may influence the prevention of HCC development [11, 95–100]. The results of a retrospective analysis showed that BCAA supplementation (12 g/day for >6 months) reduced the incidence of HCC in patients with liver cirrhosis with a hazard ratio of 0.42 (95% CI, 0.22–0.80; $p = 0.009$) [95]. Oral supplementation of BCAA (12 g/day for 6 months) significantly decreased the serum levels of AFP and reduced early recurrence after hepatic resection in patients with HCC [98]. In a subset analysis of the long-term survival study, Muto et al. also showed that long-term oral supplementation with BCAA significantly inhibited the development of HCC in type C cirrhotic patients with BMIs >25 [11]. Moreover, the administration of BCAA granules (12 g/day for 60 months) markedly inhibited the cumulative recurrence of HCC, after curative treatment in patients, with insulin resistance [96]. Therefore, long-term treatment with BCAA is an effective strategy for improving the clinical outcomes in cirrhotic patients by reducing the likelihood of liver failure and in obese and diabetic patients, by suppressing liver carcinogenesis. Pathophysiological conditions involved in the development of obesity-related HCC and in the precise mechanisms of BCAA to inhibit liver carcinogenesis, in particular the mechanisms associated with obesity, are discussed in the following sections.

11 Obesity and HCC

Among patients with liver cirrhosis, the proportion of obese subjects is gradually increasing [101, 102]. This is a serious problem when considering the medical care of chronic liver disease because obesity and its related metabolic abnormalities, especially diabetes mellitus, are major risk factors for the development of HCC [11, 103–106]. Nonalcoholic fatty liver disease, a hepatic manifestation of obesity and metabolic syndrome, is also an important healthcare problem, especially in developed countries, since it can progress to nonalcoholic steatohepatitis), which in turn leads to liver cirrhosis and HCC development [107, 108].

Recent studies have shown several pathophysiological mechanisms linking obesity and liver carcinogenesis, including the emergence of insulin resistance, activation of the IGF/IGF-1R axis, development of a state of chronic inflammation, induction of oxidative stress, and adipokine imbalance [103, 104]. In particular, insulin resistance, which leads to systemic and hepatic inflammation, liver steatosis, and activation of the IGF/IGF-1R axis, is considered to play a critical role in the development of HCC [35, 103, 104, 109]. On the other hand, these reports strongly indicate that targeting such pathophysiological disorders via pharmaceutical and nutraceutical intervention might be an effective strategy to prevent obesity-related liver carcinogenesis [16, 110]. For instance, pitavastatin, a drug widely used for the treatment of hyperlipidemia, and (−)-epigallocatechin-3-gallate, one of the green tea catechins, significantly inhibit the obesity-related liver tumorigenesis by attenuating the chronic inflammation induced by excess fat deposition [111, 112]. Administration of ACR also suppresses diethylnitrosamine-induced liver tumorigenesis in obese and diabetic mice and this is associated with inhibition of Ras activation and phosphorylation of the ERK and RXRα proteins [17]. Increase in insulin sensitivity and the attenuation of systemic and hepatic inflammation by ACR also contribute to this inhibition [17], indicating that ACR might be useful in the chemoprevention of obesity-related HCC (Fig. 2).

12 Preventive mechanisms of BCAA in obesity-related liver carcinogenesis

Recent experimental studies have revealed that BCAA improves insulin resistance and glucose tolerance via the enhancement of glucose metabolism in skeletal muscle, adipose tissue, and the liver [113–118]. Improvements in insulin resistance and glucose tolerance, by oral BCAA supplementation in chronic liver disease patients, have also been reported in several clinical trials [119–121]. In addition, a recent in vitro study showed that BCAA treatment suppresses insulin-induced proliferation of HCC cells by inhibiting the insulin-induced activation of the PI3K/Akt pathway and the subsequent antiapoptotic pathway [89]. We, therefore, consider that improvements in glucose metabolism and insulin resistance might be a critical mechanism in the reduction of the incidence of HCC development in obese cirrhotic patients [11]. This hypothesis was evaluated using an obesity- and diabetes-related liver carcinogenesis mouse model [18]. In the model, BCAA supplementation significantly inhibited diethylnitrosamine-induced liver tumorigenesis in obese and diabetic *db/db* mice by improving liver steatosis and fibrosis, insulin resistance, and hyperleptinemia [18]. Supplementation with BCAA also inhibited the spontaneous development of hepatic premalignant lesions in *db/db* mice via the attenuation of chronic inflammation in both the liver and white adipose tissue [122]. Moreover, BCAA treatment significantly inhibited the proliferation of human HCC-derived cells induced by visfatin, a serum adipokine that is significantly correlated with stage progression and tumor enlargement of HCC [90]. Yoshiji et al. [92] also reported that, in obese and diabetic rats exhibiting insulin resistance, BCAA treatment significantly exerted a chemopreventive effect against HCC through the suppression of hepatic neovascularization. The results of these reports [18, 89, 90, 92, 122] strongly indicate that BCAA inhibits obesity-related liver carcinogenesis by targeting insulin resistance and subsequently by reducing chronic inflammation and adipokine imbalance (Fig. 2). In addition to the liver, supplementation with BCAA suppressed obesity- and diabetes-related carcinogenesis in the colorectum, and this was also associated with the improvement of insulin resistance and inhibition of the activation of

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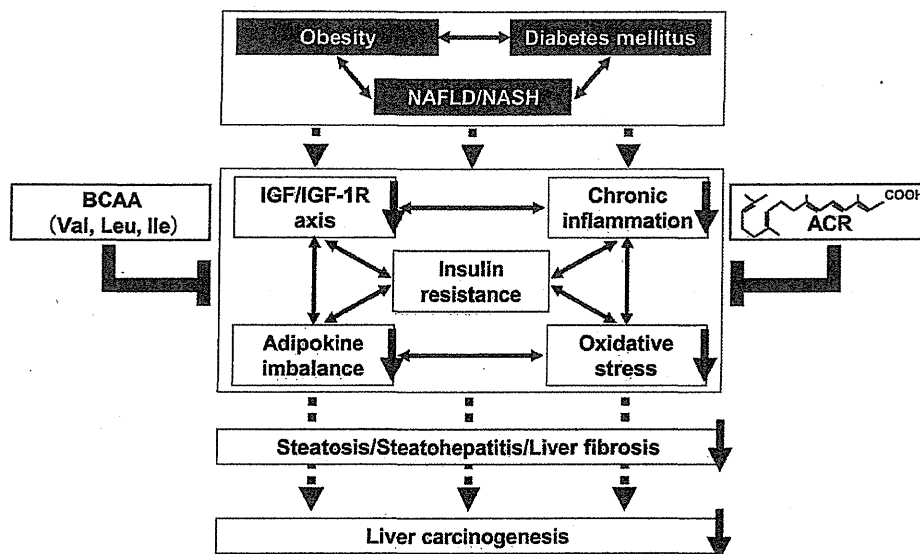

Figure 2. The mechanisms of action of ACR and BCAA in the inhibition of obesity-related liver carcinogenesis. Obesity and diabetes mellitus significantly increase the risk of HCC. Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis, which are usually associated with obesity and diabetes, also play critical roles in the development of HCC. Several pathophysiological mechanisms link obesity and liver carcinogenesis, including the emergence of insulin resistance, activation of the IGF/IGF-1R axis, a state of chronic inflammation, induction of oxidative stress, and occurrence of adipokine imbalance. Among them, in particular, insulin resistance plays a key role in obesity-related liver carcinogenesis. Oral supplementation with BCAA significantly reduces the risk of HCC development in obese cirrhotic patients, and this might be associated with decreased insulin resistance and hepatic steatosis, inhibition of the activation of the IGF/IGF-1R axis, and attenuation of oxidative stress and hyperleptinemia. ACR administration also prevents obesity- and diabetes-related liver tumorigenesis in mice by improving hepatic steatosis and insulin resistance, while attenuating chronic inflammation.

the IGF/IGF-IR axis [123]. BCAA, therefore, may be a useful chemoprevention modality for HCC and probably colorectal cancer in obese people.

13 Conclusion

Throughout this review, we have indicated that both ACR and BCAA are promising agents for the prevention of liver carcinogenesis. Therefore, we considered that a combination therapy involving both ACR and BCAA may better inhibit HCC cell growth. Interestingly, a combined ACR and BCAA treatment significantly inhibited the growth of human HCC xenografts in nude mice by inhibiting the phosphorylation of the RXRα, ERK, Akt, and IGF-1R proteins in the xenografts [124]. These results indicated that this combination might be effective for the treatment and probably chemoprevention of HCC. The beneficial effects of the combination approach to chemoprevention, using ACR as a key agent for the prevention and treatment of HCC, have been previously reported [58, 59, 125–127]. A clinical trial also demonstrated that the combination of BCAA and perindopril, an antihypertensive drug, inhibited the cumulative recurrence of HCC after curative therapy and this was associated with improved insulin resistance [128]. Therefore, a combination therapy using ACR and/or BCAA may represent a potential new strategy for chemoprevention of HCC development.

In summary, the poor prognosis of patients with HCC is because of its high incidence and recurrence in cirrhotic livers. Therefore, more effective strategies for the chemoprevention of HCC should be developed to directly improve prognoses for these patients. The results from both experimental and clinical studies strongly suggest that pharmaceutical and nutraceutical approaches, in particular using ACR and BCAA, play a central role in this strategy. These agents may also play a critical role in the prevention of obesity-related liver carcinogenesis, which is a new, serious problem in modern society.

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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.

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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

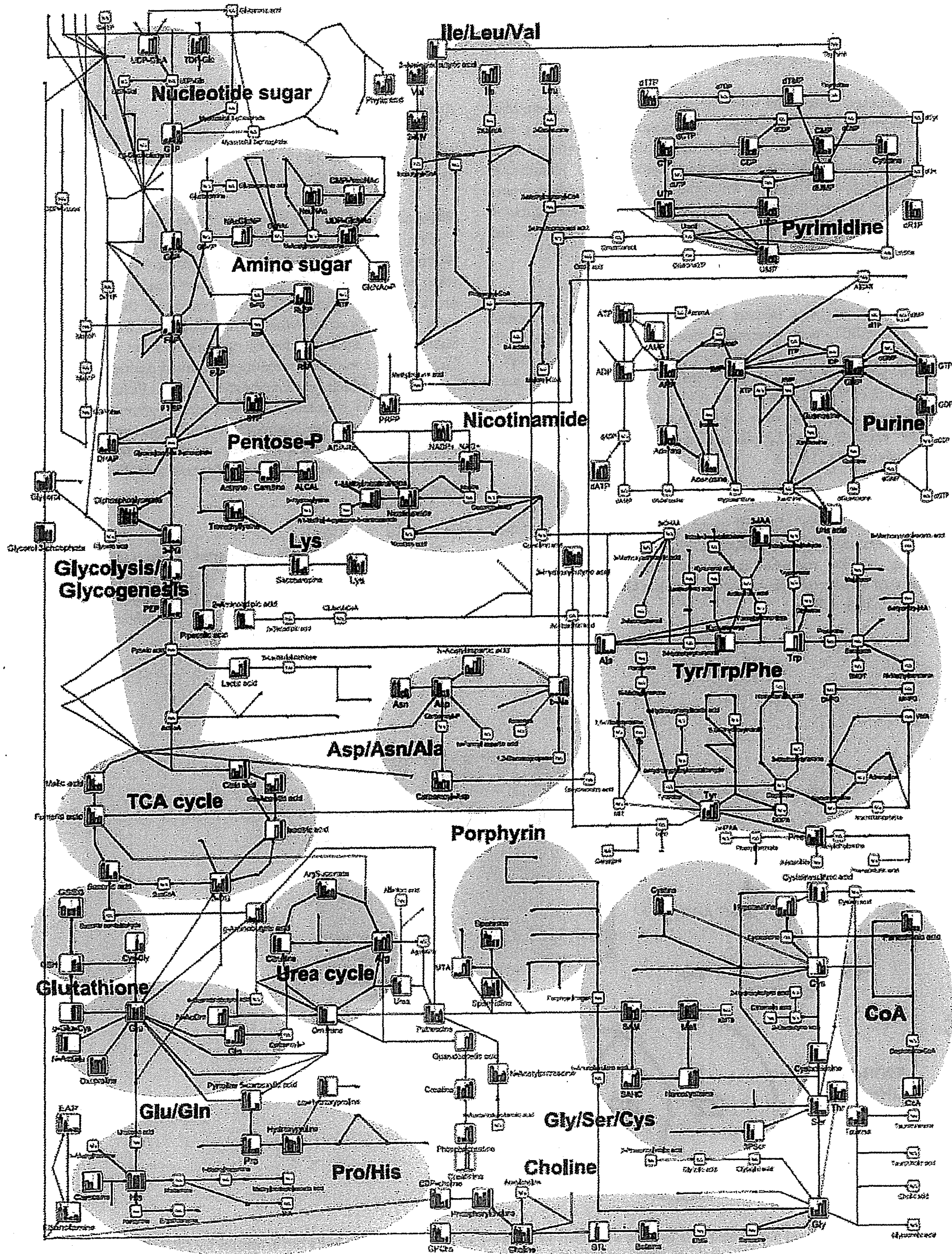

Ile/Leu/Val
Nucleotide sugar
Amino sugar
Pyrimidine
Pentose-P
Nicotinamide
Purine
Lys
Glycolysis/
Glycogenesis
Tyr/Trp/Phe
Asp/Asn/Ala
TCA cycle
Porphyrin
Glutathione
Urea cycle
CoA
Glu/Gln
Gly/Ser/Cys
Pro/His
Choline

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).

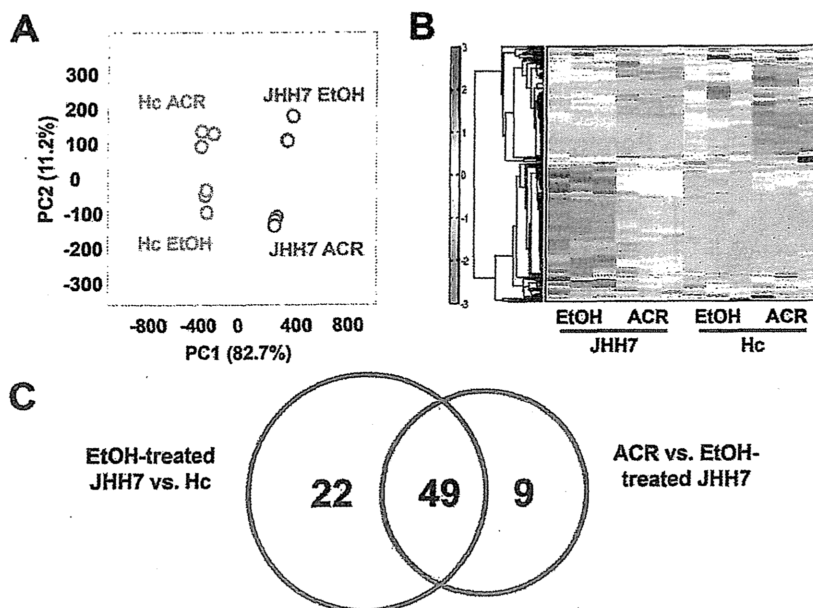
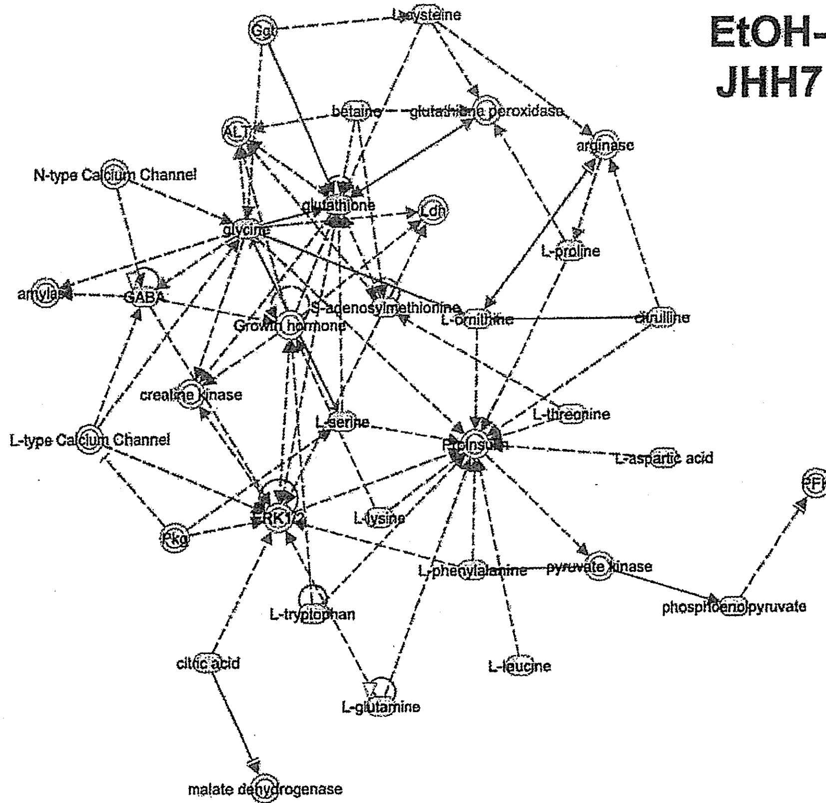


Figure 2. A comparison of the metabolic profile of EtOH- or ACR-treated JHH7 and Hc cells determined by CE-TOFMS. PCA scoreplot (*A*) and heat map (*B*) from metabolic data of JHH7 and Hc cells treated with EtOH and ACR ($n=3$). Venn-diagrams (*C*) showing the number of metabolites that were significantly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


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

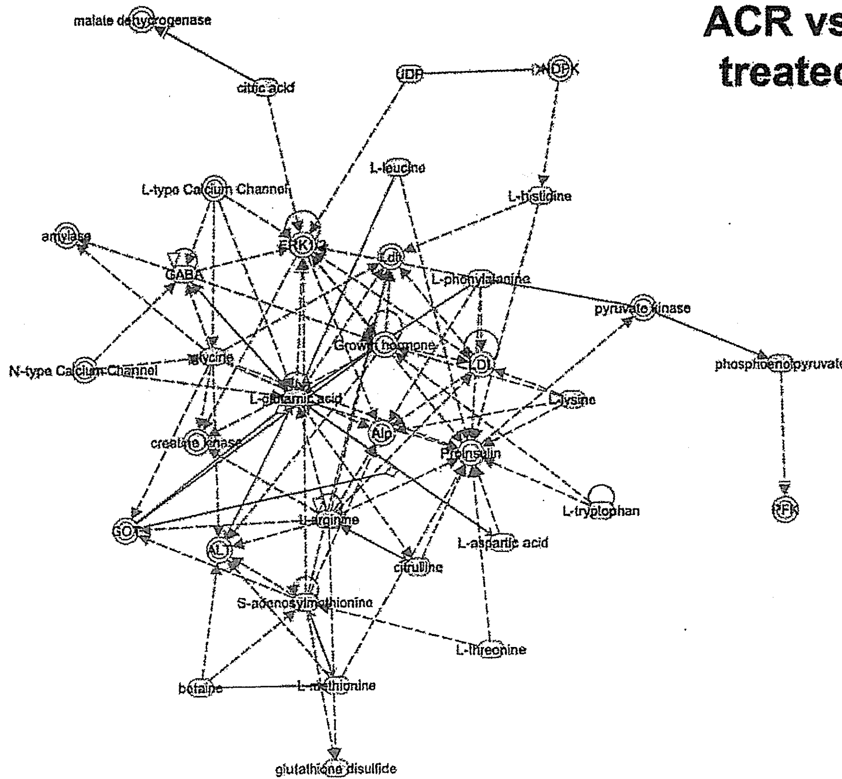

B
ACR vs. EtOH-
treated JHH7
malate dehydrogenase
citric acid
UDP
NDPK
L-leucine
L-typo Calcium Channel
L-histidine
amylase
ERK1/2
Ldh
GABA
L-phenylalanine
pyruvate kinase
Growth hormone
phosphoenolpyruvate
glycine
LDL
N-type Calcium Channel
L-glutamic acid
L-lysine
creatine kinase
Alp
Proinsulin
L-tryptophan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

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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

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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

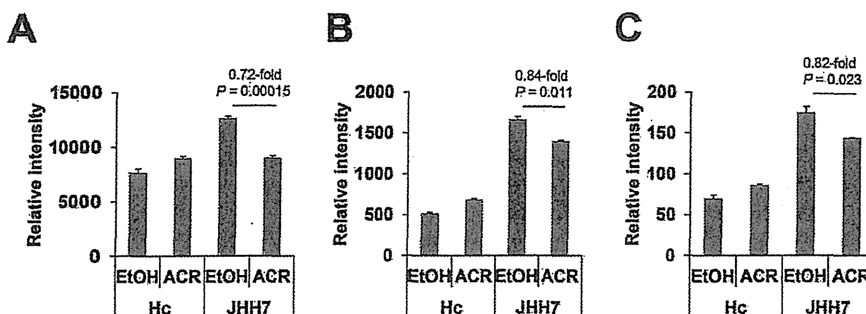

Figure 4. Levels of adenosine nucleotides in EtOH or ACR-treated JHH7 and Hc cells determined by CE-TOFMS. ATP (*A*), ADP (*B*) and AMP (*C*) levels.
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