

Prevention of Second Primary Tumors by an Acyclic Retinoid in Patients with Hepatocellular Carcinoma

Updated Analysis of the Long-Term Follow-Up Data

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Key Words

Lectin-reactive alpha-fetoprotein · Chemoprevention · Clonal deletion · Protein induced by vitamin K absence or antagonist-II · Retinoid

Abstract

Oral administration with acyclic retinoid, a synthetic vitamin A analog, for a limited period of 12 months (48 weeks) prevented the development of second primary hepatocellular carcinoma (HCC) and also improved the survival of patients who underwent curative treatments of the initial tumor. Following that randomized controlled study reported in 1996 and 1999, we have continued to follow up the patients by medical imaging and blood chemical analyses, and found that the preventive effect of acyclic retinoid lasted up to 199 weeks after randomization (or 151 weeks after completion of retinoid administration). The retinoid's effect was not mediated by reduction in hepatic necro-inflammation since no significant decrease in serum aminotransferase activity was seen in the retinoid group. Such observation seems quite distinct from the cancer-preventive mechanism of interferon, a potent immunopreventive agent for HCC. We have also shown here the reduction by the retinoid in

serum levels of lectin-reactive α -fetoprotein (AFP-L3) and protein induced by vitamin K absence or antagonist-II (PIVKA-II), both of which indicate the presence of latent HCC cells. These results suggest that acyclic retinoid may delete such malignant clones before they expand to clinically detectable tumors and thereby inhibited second primary HCC. Once such latent clones are eradicated, it may well take at least several years for the next cancer clone to arise clinically. This may possibly explain a reason for the long-term effect of the retinoid even after the limited period of administration.

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Introduction

The goal of cancer chemoprevention is either to prevent or reverse the carcinogenic process in the initiation and promotion phases before the clinical development of cancer and/or to delete premalignant or latent malignant clones from an organ by apoptosis or differentiation induction. Such cancer chemoprevention is important for a population with apparent premalignant lesions and particularly inevitable for the patients who received curative treatment of preceding cancer but still have a high risk to

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develop second primary cancer [1]. The organs exposed to a continuous carcinogenic insult such as persistent viral infection likely have multiple clones of premalignant cells that lead to multicentric carcinogenesis. Such characteristic pattern of carcinogenesis is explained by a concept of 'field cancerization' [1]. Eradication of such abnormal clones from the field is essential to prevent the second primary cancer.

Chronic infection with hepatitis type B or C virus is the major cause (90% or more) of hepatocellular carcinoma (HCC) in Japan with the annual incidence of carcinogenesis as high as 5–7% in cirrhotic patients [2]. Furthermore, the annual occurrence rate of second HCC, including second primary HCC and recurrent HCC, after the initial treatment rises to about 30% [3], accounting for the poor prognosis of the disease. Accordingly, the prevention of second tumors has become the most important strategy to improve the prognosis, in addition to efforts aimed at early detection and treatment of the second cancer.

An acyclic retinoid, or polyprenoic acid [(2*E*,4*E*,6*E*,10*E*)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid, C₂₀H₃₀O₂, molecular weight: 302.46], has agonistic activity for the nuclear retinoid receptors, retinoic acid receptor (RAR) and retinoid X receptor (RXR) [4, 5], and induces cell differentiation [4] and programmed cell death (apoptosis) [6] of HCC cells. A clinical study has shown that oral administration with the retinoid for 48 weeks resulted in significant inhibition of second primary HCC after radical treatments [7] and subsequent improvement of survival [8]. Furthermore, acyclic retinoid was safe, as it did not lead to the adverse reactions that are seen with other retinoids [7]. Following that clinical study reported in 1996 and 1999, we have attempted to elucidate the underlying mechanism of the retinoid's cancer-preventive effect. We here show that the retinoid inhibited the evolution of second transformed clones, by measuring serum levels of both an isoform of α -fetoprotein (AFP) and protein induced by vitamin K absence or antagonist-II (PIVKA-II) as possible biomarkers of such clones. AFP has a microheterogeneity due to structural variations in its sugar chain [9]. AFP-L3, an isoform of AFP, can be identified due to its reactivity with *Lens culinaris* agglutinin. Both AFP-L3 and PIVKA-II are known to suggest the presence of latent HCC cells in the liver in advance of the detection by image diagnosis [9–12]. We have also found that the retinoid was effective up to 199 weeks including the initial administration term of 48 weeks. The eradication of latent HCC clones may possibly be the mechanism of the long-lasting cancer chemoprevention by acyclic retinoid.

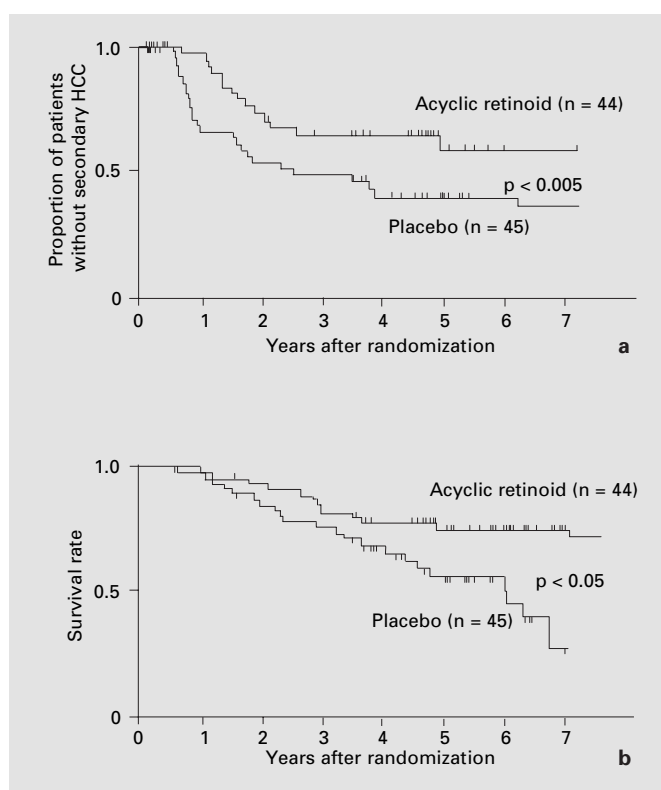


Fig. 1. Kaplan-Meier estimates of the disease-free survival (a) and absolute survival (b) in patients with previously treated tumors who were given acyclic retinoid or placebo. Drug administration lasted from 0 to 1 year (48 weeks). Difference between the two curves was examined statistically by the log-rank test.

Patients and Methods

Patients

The study involved 89 patients who were enrolled in the clinical trial to prevent second primary HCC by acyclic retinoid at Department of Gastroenterology, Gifu University School of Medicine and its five affiliate hospitals [7]. Every patient received curative surgical resection or ultrasonography-guided percutaneous ethanol ablation therapy of the initial HCC, and was confirmed to be free of further tumor by ultrasonography and X-ray computed tomography (CT). The clinical profiles, laboratory data, and characteristics of preceding HCC were reported previously [7]. All patients gave written informed consent before participating in this study. This study was approved by each institution's review board for human research.

Study Design

The patients were assigned randomly to either the acyclic retinoid group (n = 44) or the placebo group (n = 45). Patients in the acyclic retinoid group received an oral administration of 600 mg of the compound daily for 48 weeks, and those in the placebo group received a placebo capsule that contained vehicle only. Every patient was examined by ultrasonography every 3 months and by CT or magnetic resonance images (MRI) every 6 months in both the drug administration

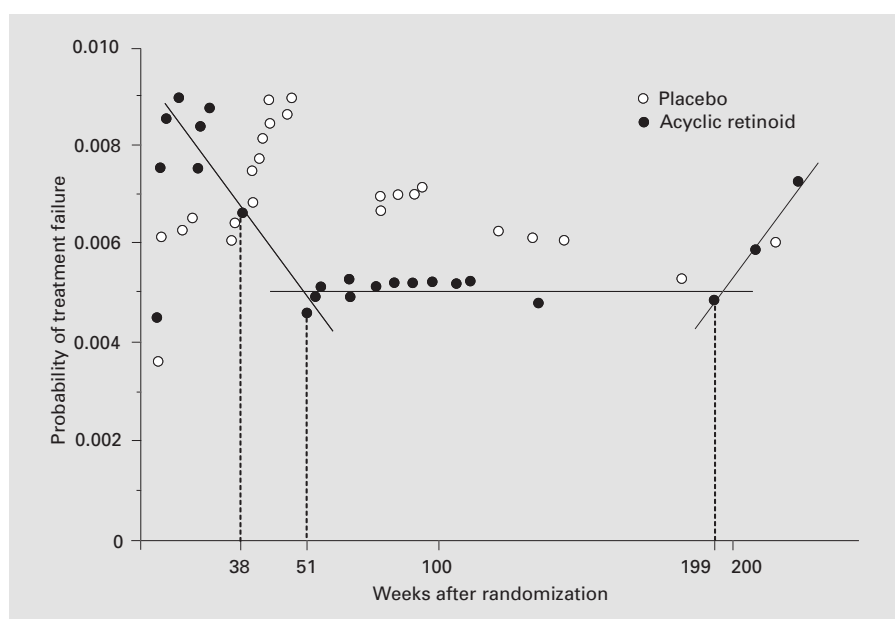


Fig. 2. Weibull estimates of probability of treatment failure in placebo (○) and acyclic retinoid (●) groups.

period and the subsequent follow-up period. In parallel blood biochemistry was examined at every visit. The detailed protocol of the study has been published previously [7].

Determination of Serum Levels of AFP-L3 and PIVKA-II

Serum samples obtained from 42 patients at study entry and at the end of the 48-week period of the drug administration were subjected to determination of total AFP and PIVKA-II levels using chemiluminescence immunosorbent assay kits (Lumipulse AFP N, Fuji Rebio, Tokyo, and Picolumi PIVKA-II, Eisai, Tokyo, Japan, respectively). AFP-L3 was determined by lectin-affinity electrophoresis coupled with antibody-affinity blotting, employing a commercial kit (AFP Differentiation kit L, Wako Pure Chemical, Osaka, Japan).

Statistical Analysis

Differences between AFP-L3 or PIVKA-II levels at entry and those after 48-week treatment period were examined statistically by the paired t test. Incidences of AFP-L3- or PIVKA-II-positive patients were compared between the two groups using Fisher's exact test. Survival curves were estimated by the method of Kaplan and Meier, and differences between two curves were examined by the log-rank test. Survival curves were then fitted to Weibull distribution, and probability of treatment failure was estimated at each censored time point. All statistical analyses were performed on StatView ver 4.5 (Abacus Concepts, Berkeley, Calif., USA). All data were updated in Oct 2003.

Results

We first updated the treatment failure as well as survival of the total 89 patients (fig. 1). There was no statistically significant difference in demographic and clinical

characteristics of the patients at entry between the two groups, which might influence the occurrence of second primary HCC, as reported previously [7]. Second primary HCC developed in 23 and 28 patients during the study period for 7 years in the acyclic retinoid and the placebo group, respectively. We have already shown that administration of acyclic retinoid prevented significantly the development of second primary HCC in this chemoprevention trial in 1996 [7] and also improved the survival significantly in 1999 [8], both of which are further confirmed in the present study. It is notable that administration of the retinoid for only 48 weeks seems to result in such beneficial effects for as long as several years.

Thus, to assess the effective term of the retinoid to prevent second HCC, we analyzed probability of treatment failure at each censored time-point (fig. 2). The probability was not different between placebo and retinoid groups in the early phase up to 38 weeks after randomization. However, the probability became significantly lower in the retinoid group between 51 and 199 weeks (95% confidence interval, 180–233 weeks), suggesting that this period was the effective term of the retinoid. After 199 weeks, the probability began to increase in the retinoid group.

Recently, a number of large-scale clinical studies have also shown the preventive effect by interferon (IFN), a potent immunopreventive agent of HCC [14, 15]. Since IFN is known to suppress hepatic necroinflammation and thereby to prevent hepatocarcinogenesis, we next exam-

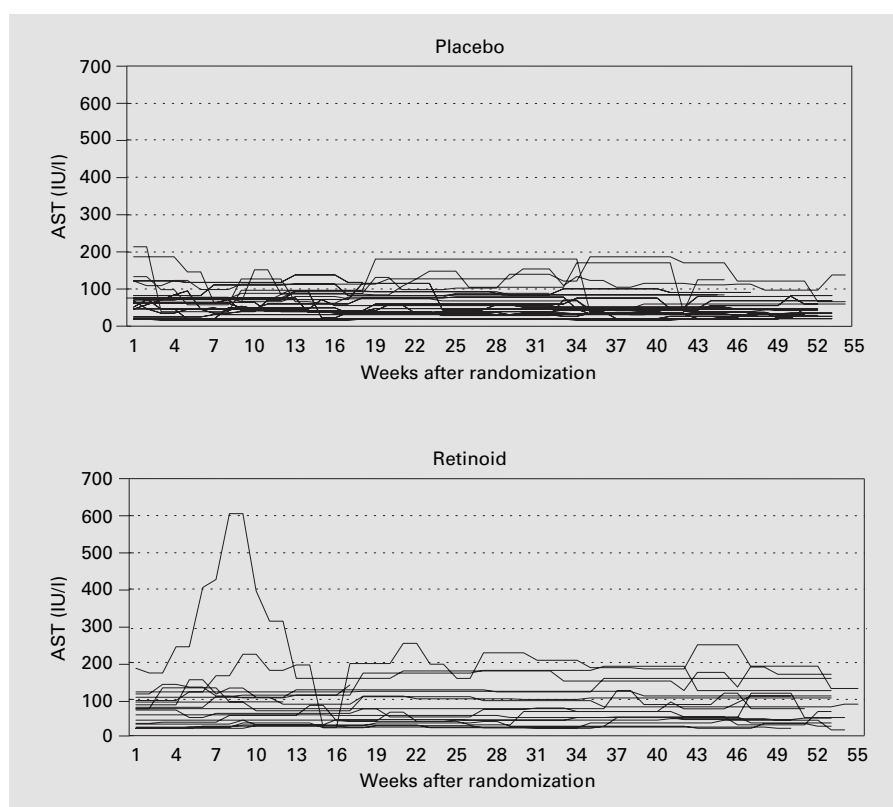


Fig. 3. Time-course alterations of serum activities of AST in each patient who was given placebo (upper panel) or acyclic retinoid (lower panel).

Table 1. Mean laboratory data during the observation period

	Placebo	Acyclic retinoid
Alb, g/dl	4.1 ± 0.4	4.1 ± 0.5
T-Bil, mg/dl	0.8 ± 0.3	0.7 ± 0.3
AST, IU/l	66 ± 32	82 ± 47
ALT, IU/l	60 ± 35	88 ± 52
ALP, IU/l	223 ± 48	248 ± 70
γ-GTP, IU/l	76 ± 60	70 ± 41
BUN, mg/dl	16.6 ± 6.6	15.9 ± 3.7
T Chol, mg/dl	159 ± 34	157 ± 29
TG, mg/dl	92 ± 40	127 ± 55
WBC, /μl	4,717 ± 1,520	4,497 ± 1,689
Plt × 10 ⁴ /μl	13.4 ± 6.9	14.0 ± 5.9
PT, %	80 ± 12	85 ± 12
HPT, %	75 ± 14	67 ± 21
NH ₃ , μg/dl	91 ± 28	66 ± 28

Values represent the mean ± SD (n = 21 in both groups). The average value in each patient was calculated from the data in each month during the observation period, from which the mean value of all the patients was obtained in the respective group.

ined if the retinoid might have similar effect. We analyzed 42 patients (21 each in placebo and retinoid groups) whose clinical courses could be followed up in detail during the observation period [13]. Every patient was examined at least once a month by biochemical analyses. However, there was no significant difference in the average values of any liver function tests, including serum activities of aminotransferases, between the placebo and retinoid groups (table 1). The time-course alterations of serum AST activities in both groups are depicted in figure 3, demonstrating that the retinoid did not affect hepatic necroinflammation. These observations suggest that a cancer-preventive mechanism of acyclic retinoid is distinct from that of IFN.

We have previously reported a direct effect of the retinoid on the HCC cells, i.e. eradication of malignant clones from the remnant liver by inducing apoptosis and/or differentiation [4, 6]. To confirm such clonal deletion mechanism, we examined if the retinoid suppress serum levels of AFP-L3 and PIVKA-II, potent biomarkers indicating the presence of latent cancer cells in the liver that escape the detection limit by image diagnosis. At entry, no significant difference was found in mean serum levels of either

AFP-L3 (1.5 ± 3.4 and 2.2 ± 8.2 ng/ml in the placebo and retinoid groups, respectively, the mean \pm SD) or PIVKA-II (25.9 ± 17.8 and 19.4 ± 10.4 mAU/ml in the placebo and retinoid groups, respectively) between the groups. The incidence of AFP-L3-positive patients rose significantly ($p < 0.05$) from 4/21 (19%) to 12/21 (57%) and the serum levels of AFP-L3 elevated significantly ($p < 0.01$) after 48 weeks in the placebo group (table 2). In contrast, both the incidence of AFP-L3-positive patients (5/21 at entry and 1/21 at 48 weeks, $p < 0.1$) and the serum levels of AFP-L3 were reduced in retinoid group ($p < 0.01$) (table 2). Thus, the number of AFP-L3-positive patients after 48 weeks was significantly ($p < 0.01$) smaller in acyclic retinoid group than in placebo group (table 2). Similarly, acyclic retinoid prevented both increase in the serum levels and appearance of patients with elevated PIVKA-II levels (table 2). The detection of AFP-L3 in the serum even at faint levels suggests the presence of latent HCC [9–11, 13]. However, low levels of PIVKA-II (normal range, <40 mAU/ml) do not always suggest such latent tumors. Hence, we defined a patient to be PIVKA-II-positive in case his serum level increased by 1.5-fold or more at 48 weeks compared to that at the entry. Both the incidence of such PIVKA-II-positive patients and the mean serum levels were lower in the retinoid group at 48 weeks (table 2). Consequently, the number of patients with both AFP-L3-positive and increased PIVKA-II was significantly ($p < 0.05$) lower in the retinoid group (0/21, 0%) compared to the placebo group (5/21, 24%) (table 2).

The proportion of patients free of second primary HCC in the follow-up period was significantly higher in the patients with negative AFP-L3 than in the positive group after 48 weeks, as reported previously ($p < 0.03$) [13]. In a similar manner, PIVKA-II at 48 weeks also produced significant differences in both disease-free survival and absolute survival (fig. 4). By Cox's proportional hazards model analysis, AFP-L3 and PIVKA-II were significant independent factors to predict disease-free survival [relative risk (RR) 0.53, $p = 0.018$, and RR 0.39, $p = 0.003$, respectively] and absolute survival (RR 0.50, $p = 0.039$, and RR 0.52, $p = 0.029$, respectively).

Discussion

The present study confirmed the long-term effect of acyclic retinoid to prevent second primary HCC, which lasted for about 150 weeks even after completion of drug administration for 48 weeks (fig. 1, 2). Such an effect was not mediated by the suppression of hepatic necroinflam-

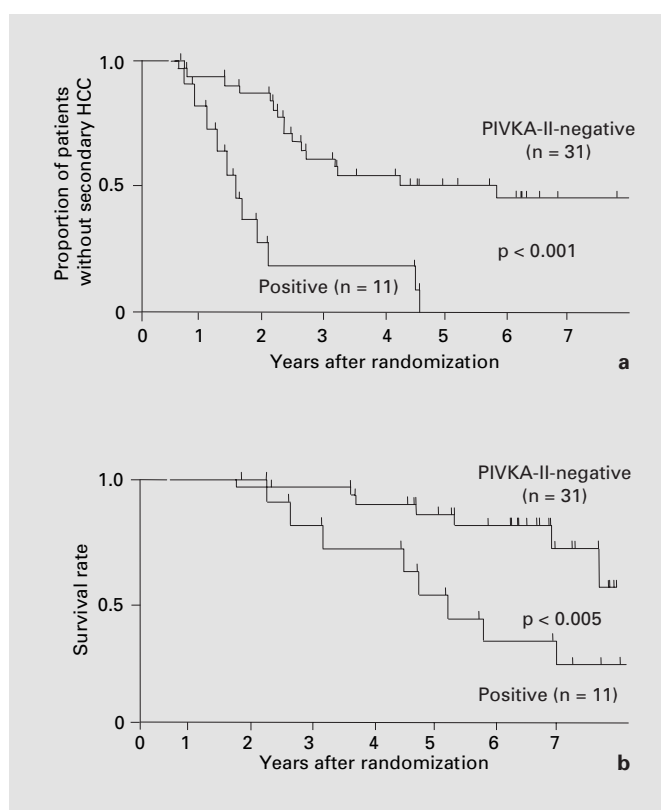


Fig. 4. Kaplan-Meier estimates of disease-free survival (a) and absolute survival (b) in patients with positive and negative PIVKA-II after 48 weeks of drug administration. A patient was defined as PIVKA-II positive when his serum level increased by 1.5-fold or more at 48 weeks compared with that at the entry.

Table 2. Number of patients with elevated tumor biomarkers after 48 weeks of drug administration

	Placebo	Acyclic retinoid
Increased AFP-L3		
Number of patients	12/21	1/21**
Serum levels, ng/ml	13.6 ± 20.1	$0.02 \pm 0.09^{**}$
Increased PIVKA-II		
Number of patients	6/21	2/21
Serum levels, mAU/ml	70.2 ± 14.1	$13.6 \pm 20.1^*$
Both markers increased		
Number of patients	5/21	0/21*

Values represent the mean \pm SD after 48 weeks of administration ($n = 21$ in both groups).

* $p < 0.05$ and ** $p < 0.01$ compared to the values of placebo group.

mation (table 1; fig. 3) but by the direct eradication of the latent malignant clones as suggested by the downregulation of AFP-L3 and PIVKA-II (table 2; fig. 4).

We have previously proposed a concept of 'clonal deletion' as a mechanism of chemoprevention of HCC by acyclic retinoid [13]. We define 'clonal deletion' therapy as a removal of latent malignant (or premalignant) cells from an organ with hypercarcinogenic state [16]. It is very likely that cirrhotic livers have latent malignant cell clones particularly after treatments of the initial HCC as suggested by the extremely high annual incidence of recurrence [3]. Although such latent clones are hardly detected in clinical practice by image diagnosis, it is suggested that AFP-L3 and PIVKA-II may recognize them and predict the occurrence of HCC at the clinical level [9–11]. Therefore, suppression of these serum biomarkers shown in the present study may well suggest the deletion of latent malignant clones. Once such clones are eradicated from the liver, it takes at least several years for de novo carcinogenesis, which may be one of the reasons for the long-term benefit of acyclic retinoid even after the completion of its drug administration.

In general, two possible mechanisms are widely accepted for such removal of transformed cells, i.e. cell death (or apoptosis) and differentiation induction. These two mechanisms may work concurrently in some systems. We have shown the induction of both differentiation [4] and apoptosis [6] by acyclic retinoid in HCC cells. Acyclic retinoid upregulated albumin (a marker of mature hepatocytes) whereas it downregulated AFP (a marker of immature or transformed hepatocytes) expression, suggesting a differentiation in the induction of HCC cells [4]. Further information is now accumulating to show the phenotypic changes of HCC cells, including suppression of telomerase activity and other sequential genetic alterations that are typical for mature hepatocytes [6].

We have also suggested that loss of retinoid signaling to maintain the normal cell function seems to be linked to the development of HCC. We have found a malfunction of nuclear retinoid receptor in HCC cells. Among retinoid receptors, RXR- α is most abundant in the liver and highly expressed in HCC cells. Recently, we have reported that RXR- α is phosphorylated by extracellular signal-regulated kinase 1/2 and accumulated in HCC cells [17, 18]. We found that phosphorylation interferes with the transactivating activity of RXR- α via its response elements. We have suggested that some RXR- α -related genes are involved in cell growth arrest and/or apoptosis induction, including p21 [19] and STAT1 [20]. Thus, interference with the induction of such genes might render the cancer

cells resistant to RA-induced apoptosis. Moreover, phospho-inactivated RXR- α is sequestered from proteolytic degradation via ubiquitin/proteasome-mediated pathway, resulting in a dominant-negative accumulation of the non-functioning receptor in the HCC cells [20]. Very recently, we have found that acyclic retinoid not only functions as an RXR- α -ligand but also suppresses phosphorylation of RXR- α by inactivating Ras/Erk system [21]. This novel molecular effect of acyclic retinoid may give us a key to unveil the unknown mechanism of its specific antitumor activity against HCC.

Because the removal of such malignant clones could be recognized as a therapy rather than disease prevention, these ideas may allow us to place chemoprevention in a similar category as chemotherapy [16]. In addition, because the mechanisms by which acyclic retinoid prevent hepatocarcinogenesis seems to be distinct from those of IFN, it is possible that these agents exert synergistic effect when used in combination. In fact, we have reported the cooperative effect of the retinoid and IFN to induce apoptosis of HCC cells [20], suggesting a future benefit of biochemoprevention by their combination [16].

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