

## Treatment with All-trans Retinoic Acid Plus Tamoxifen and Vitamin E in Advanced Hepatocellular Carcinoma

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**Abstract.** *Background:* Low serum retinol and hepatic tocopherol levels correlate with hepatocellular carcinoma (HCC) risk. Antiestrogen tamoxifen seems useful in HCC patients. A pilot study was performed to evaluate the effect of all-trans retinoic acid associated with tamoxifen and vitamin E on patients with advanced HCC. *Patients and Methods:* Fifteen consecutive patients with advanced HCC were included in the study. Patients were evaluated for survival, quality of life, liver function, tumor mass, toxicity related to the treatment and retinoid receptors in liver biopsies. *Results:* The median survival of our patients was 22 months. Pain and asthenia were improved in the majority of patients. Every patient with baseline elevated liver enzymes showed an improvement in liver function. RAR- $\alpha$ , RXR- $\alpha$ , RAR- $\beta$  and RAR- $\gamma$  receptors were demonstrated in 100%, 73%, 47% and 40%, respectively. *Conclusion:* A combination therapy of all-trans retinoic acid, tamoxifen and vitamin E increases the survival rate and ameliorates the clinical outcome in patients with inoperable HCC.

*Abbreviations:* HCC, hepatocellular carcinoma; CLIP, the Cancer of the Liver Italian Program; VAS, visual analogue scale; CT, computed tomographic; ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase;  $\gamma$ -GT, gamma-glutamyltranspeptidase; ALP, alkaline phosphatase; AFP,  $\alpha$ -fetoprotein; RAR, retinoic acid receptor; RXR, retinoid X receptor; MHC, major histocompatibility complex; MICA/B, class I-related chain A/B; PCR, polymerase chain reaction; TR, thyroid hormone receptor; VDR, vitamin D receptor; PPAR, peroxisome proliferator activated receptor; LXR, liver X receptor; FXR, farnesoid X receptor; BSEP, bile salt export pump; FXREs, farnesoid X receptor response elements; BAREs, bile acid receptor response elements; QoL, quality of life.

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*Key Words:* Hepatocellular carcinoma, retinoic acid, tamoxifen, RAR receptors.

Hepatocellular carcinoma (HCC) is one of the ten most common cancers in the world (1), with an estimated annual incidence of about 1,000,000 cases (2,3). Treatments of HCC are disappointing, especially in patients with cirrhosis whose prognosis is determined not only by the characteristics of the liver cancer but also by the underlying liver disease and the degree of liver failure. When the early symptoms are recognized, most of the patients have inoperable HCC (4). The median survival of HCC untreated patients with an Okuda II stage and with a Cancer Liver Italian Program Investigation (CLIP) score of 2-3 was about 7 months. In patients with Okuda stage III and CLIP scores of 4-6 it was 3 months (5,6). Chemotherapy with doxorubicine, epirubicine, tegatur or cisplatin did not increase the survival and induced fatal toxicity in 25% of the patients (7). Lipiodol chemoembolization reduced tumor growth but often caused acute liver failure and did not significantly improve survival (8). The presence of estrogen receptor in the liver and the supposed causative relationship between sex steroids and liver tumors (9,10) suggested the possibility of using the antiestrogen tamoxifen for the treatment of HCC. Tamoxifen may reduce the cytosolic and nuclear estrogen receptor-independent mechanism of tumor growth, as suggested by the absence of detectable estrogen receptor in several tamoxifen-sensitive hepatoma cell lines (11). Consistent with this view, *in vitro* and *in vivo* preclinical evidence shows that all-trans retinoic acid, an analog of vitamin A, was able to inhibit proliferation and induce apoptosis in human hepatoma cells (Hep3b) and suppress tumorigenicity in a nude mice model (12,13). Moreover, 13-cis retinoic acid reduces the incidence of 3'-methyl-4-dimethylaminobenzene-induced hepatic tumors, while retinyl acetate has a protective effect against spontaneous hepatic adenoma in mice (14,15). Epidemiological evidence showed that low serum retinol and hepatic tocopherol levels correlate with HCC risk (16,17), suggesting a potential role of retinoids for the

Table I. Patient characteristics.

N.	Initials	Sex	Age (yr)	Cause of liver cirrhosis	CLIP score	Okuda stage	Histology	Number of lesions	Survival (months)
1	SE	M	72	Alcoholic	5	III	HCC-T	Multifocal	10
2	PM	M	78	HCV	5	III	HCC-N	3	12
3	FM	M	78	HCV	2	II	HCC-N	7	24
4	CG	M	77	HCV	1	I	HCC-T	3	6
5	BA	M	63	HBV	2	II	HCC-N	4	26
6	TM	M	66	HCV	1	I	HCC-T	3	22
7	RM	M	66	HCV	2	II	HCC-N	3	10
8	BR	M	79	HCV	1	II	HCC-N	Diffuse	11
9	ML	M	68	HCV	2	II	HCC-N	1	18
10	TC	F	77	HCV	1	I	HCC-N	Diffuse	26
11	PD	F	72	HCV	2	I	HCC-N	3	24
12	FO	M	73	HBV	3	II	HCC-N	1	24
13	BF	M	69	Alcoholic	3	II	HCC-N	3	41
14	BM	M	66	Alcoholic	4	II	HCC-N	3	31
15	BA	M	63	Alcoholic	3	III	HCC-N	1	16
Mean	(M:F) 13:2		71.1		2.5	II	(T:N) 3:12		20.1
Median									22

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; HCC-T, trabecular hepatocellular carcinoma; HCC-N, nodular hepatocellular carcinoma.

chemoprevention of this cancer. Furthermore, the acyclic retinoid polyprenonic acid induces differentiation and apoptosis and prevents relapse of the primary tumor in surgical patients after surgical resection or ethanol ablation of the original one (18). Because *in vitro* and *in vivo* studies on different cancer models have demonstrated a potential antineoplastic effect of a combination of retinoids and tamoxifen (19,20), we designed a prospective pilot study to evaluate a combination therapy of tamoxifen, all-trans retinoic acid and vitamin E in patients with inoperable HCC.

Clinical measures to evaluate the effectiveness of this therapy included : a) increment of mean patients' survival relative to Okuda stage and CLIP score, b) absence of acute and sub-acute toxicity, c) patient compliance, and d) improvement of hepatic function parameters and/or tumor markers.

### Patients and Methods

**Patients.** Fifteen consecutive patients (13 male, 2 females, mean age: 71.1 ± 5.7 years) with inoperable HCC were enrolled from January 1998 to May 2002. The patient characteristics are summarised in Table I. The diagnosis of HCC was made by histological diagnosis after eco-guided biopsy. Tumors were considered inoperable based on ultrasonography, computed tomographic scan (CT) and angiography. Patients had had no previous surgical resection or antitumoral therapy; Karnovsky

score was greater than 70% (*i.e.*, the patient was ambulatory and able to take care of most daily needs). Liver biopsies were obtained to assay RAR expression levels by semi-quantitative RT-PCR.

**Trial protocol.** Informed consent was obtained from each patient and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. The protocol was approved by the University of Perugia Human Research Committee, Italy. All eligible patients were assigned to receive all-trans retinoic acid (supplied by Hoffman La Roche, Basel, Switzerland) 25 mg/m<sup>2</sup> *per os* on alternate days associated with the tamoxifen (Nolvadex; AstraZeneca) 20 mg/day *per os* and vitamin E (Ephynal, Hoffmann La Roche) 300 mg/day *per os*. Treatment continued throughout disease progression or until there was unacceptable toxicity. All patients were followed on a monthly basis for clinical assessment of general status, liver size and laboratory tests. Measures performed included full blood count, serum liver and renal biochemistries and serum a-fetoprotein (AFP). Survival time was defined as the time interval between the initiation of therapy and death. Tumor size was assessed at 4-week intervals by ultrasound and after 12 weeks by CT. Every month, any side-effects reported were recorded and asthenia and pain were assessed by a visual analogue scale (VAS). The VAS was based on a standard 10 cm line where the patients recorded their relative level of asthenia and pain (21).

**Statistical analysis.** Data regarding the parameters under study are reported as the mean ± standard error (SE). The Student's *t*-test was used to compare continuous variables between groups. The level of significance was set at *p* < 0.05.

Table II. Patient survival by CLIP score and Okuda stage.

	Median survival (months)	10-year survival	2-year survival
CLIP score			
0	35.7	84%	65%
1	22.1	66%	45%
2	8.5	45%	17%
3	6.9	36%	12%
4 to 6	3.2	9%	0%
Okuda stage			
I	23.4	68%	48%
II	6.7	36%	13%
III	2.9	21%	10%

## Results

The mean duration of treatment was 20 months. The median survival of the patients with inoperable HCC with a median CLIP score of 2-3 and a median Okuda stage of II was 22 months (Table I). This is considerably longer than the median survival times reported by both Okuda and the CLIP study where patients with a similar stage of disease had a maximum median survival of no more than 8.5 months (Table II). In patients with HCC one of the fundamental parameters is the quality of life (QoL). In our study no patient reported side-effects sufficiently severe to cause withdrawal from the study. Mild headache was observed in two patients during the first weeks of treatment, while no case of muco-cutaneous toxicity was observed. Pain and asthenia were improved in the majority of patients (87%), according to the visual-analogue scale score. After 5 and 10 months, we observed a reduction of pain by 66% and 75%, respectively, and a reduction of asthenia by 54% and 67%, respectively (Figure 1). At baseline all patients showed an increase in the parameters of hepatonecrosis and cholestasis. In particular, average values before therapy were: ASAT  $216\text{UI/L} \pm 9.5\text{SE}$  (normal value  $<40\text{UI/L}$ ), ALAT  $144\text{UI/L} \pm 5.2\text{SE}$  (normal value  $<40\text{UI/L}$ ), total bilirubin  $4.56\text{mg/dl} \pm 0.15\text{SE}$  (normal value  $<1.20\text{mg/dl}$ ),  $\gamma\text{-GT}$   $87.6\text{UI/L} \pm 3.8\text{SE}$  (normal value  $<40\text{UI/L}$ ) and ALP  $563.2\text{UI/L} \pm 11.7\text{SE}$  (normal value  $<320\text{UI/L}$ ). Administration of the combined therapy resulted in a dramatic improvement of ASAT  $67.4\text{UI/L} \pm 4.8\text{SE}$  (-68.8%,  $p < 0.05$ ), ALAT  $65.2\text{UI/L} \pm 2.6\text{SE}$  (-54.7%,  $p < 0.05$ ), total bilirubin  $2.88\text{mg/dl} \pm 0.16\text{SE}$  (-36.8%,  $p < 0.05$ ),  $\gamma\text{-GT}$   $53.2\text{UI/L} \pm 1.9\text{SE}$  (-39.3%,  $p < 0.05$ ) and ALP  $307.2\text{UI/L} \pm 13.6\text{SE}$  (-45.4%,  $p < 0.05$ ) after one month (Figures 2 and 3). Two patients with elevated AFP at entry to the study showed a 50% reduction after one month of therapy. Albumin, fibrinogen, prothrombin time, platelets and white and red blood cells levels were not changed by the therapy.

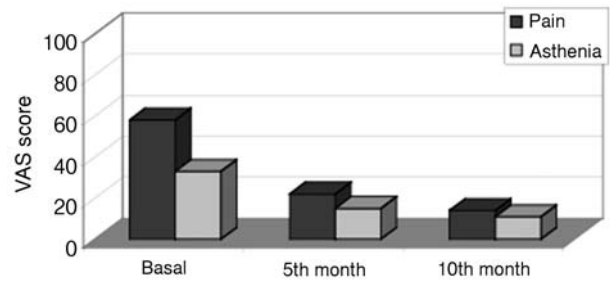


Figure 1. ATRA plus tamoxifen and vitamin E in advanced HCC: general clinical conditions in 13 symptomatic patients at baseline. Abbreviations: VAS, visual analogue scale.

The total mass and number of focal lesions remained substantially stable. Retinoid receptors were detected in all liver biopsies analyzed. RAR- $\alpha$  was present in 100% of the patients, while RAR- $\beta$  was detected in 47% of the biopsies and RAR- $\gamma$  in 40%. RXR- $\alpha$  was present in 73% of the biopsies (Table III). No significant correlation was observed between the levels of the various retinoid receptors and clinical response to the therapy.

## Discussion

The results of this study show an increase in survival in patients with inoperable HCC treated with the combination all-trans retinoic acid plus tamoxifen and vitamin E. The increment in survival in the patients treated with the combination therapy may be due to an effect of retinoids in modulating cell development, proliferation and differentiation (22). A wealth of data supports a modulatory role of retinoids and, in particular, all-trans retinoic acid, in chemical hepatocellular carcinogenesis and cancer chemoprevention. Its cellular effects are mediated by nuclear retinoid receptors. Retinoid receptors activate gene expression directly by binding to retinoic acid responsive elements within gene promoters and indirectly by interacting with other transcription factors. All-trans retinoic acid induces apoptosis in HEPG2 cells by the Jun-N-terminal kinase activation, an important signal transduction pathway in hepatoma cells (23). In a recent study it has been shown that retinoic acid induces MHC class I-like ligands MICA/B expression on human HCC and enhances natural killer cell-mediated immune responses to hepatoma cells (24). In our patients we observed a considerable reduction of cholestasis and hepato-necrosis parameters.

An interesting observation of our study was the demonstration that RXR is expressed in approximately 80% of the patients with HCC. RXR is a member of the intracellular receptor super-family and is the common

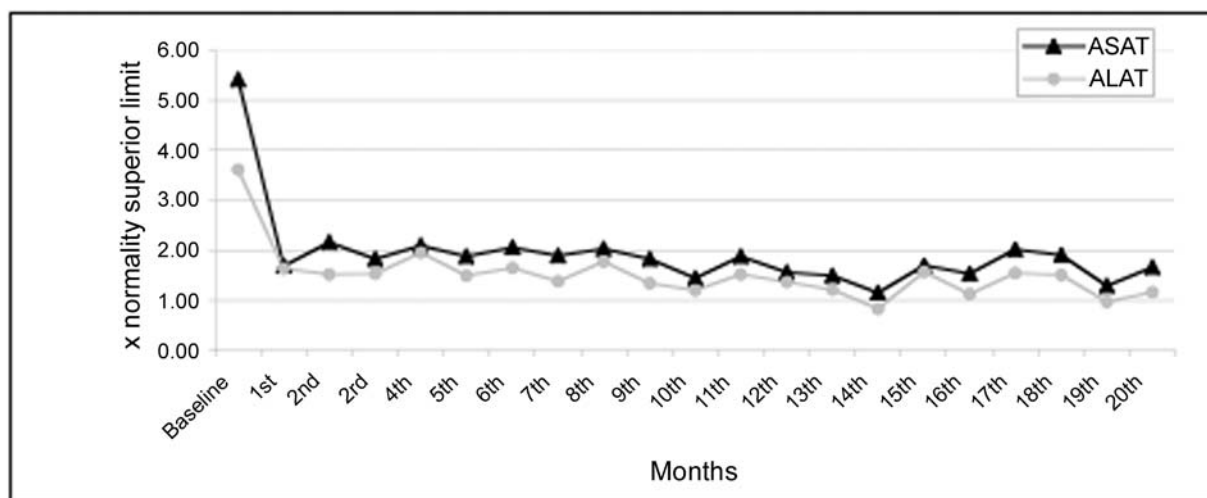


Figure 2. ATRA plus tamoxifen and vitamin E in advanced HCC: biochemical response. Abbreviations: ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase.

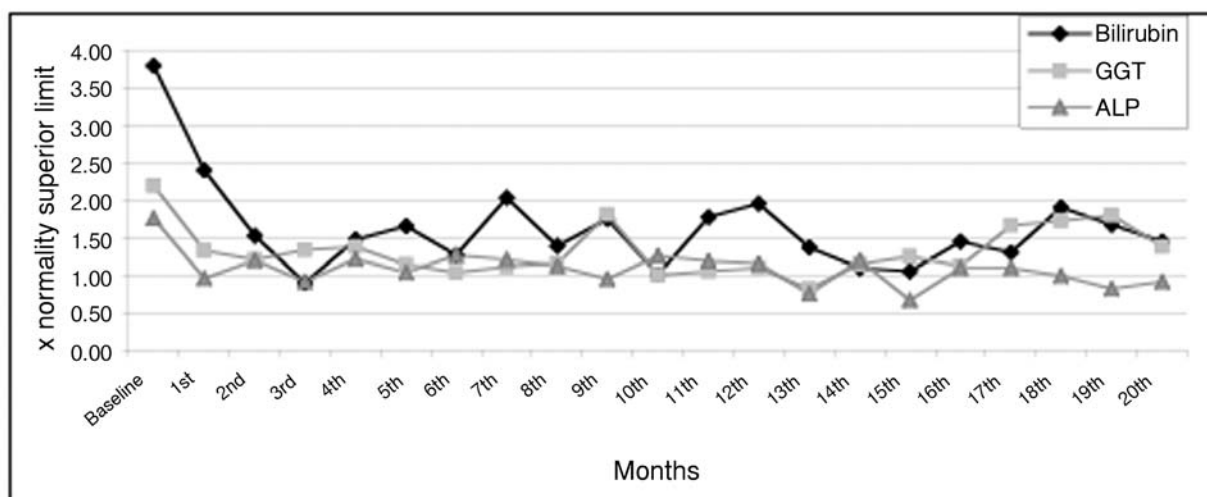


Figure 3. ATRA plus tamoxifen and vitamin E in advanced HCC: biochemical response. Abbreviations: GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase.

heterodimeric partner for many receptors including the thyroid hormone receptor (TR), retinoic acid receptor (RAR), vitamin D receptor (VDR), peroxisome proliferator activated receptor (PPAR), liver X receptor (LXR) and farnesoid X receptor (FXR) (25,26). Bile acids serve as natural ligands for FXR, which control many aspects of bile acid synthesis and transport. *In vitro* studies have shown that the FXR/RXR heterodimer binds to farnesoid X receptor response elements (FXRE) or bile acid response elements (BARE) found in the promoter of FXR-responsive genes such as the bile salt export pump (BSEP) which controls the excretion of bile acids from hepatocytes (27). The BSEP

gene promoter is stimulated by bile acid-bound FXR and transactivation by FXR/RXR is lost when this FXRE is mutated (28). Since RXR is the heterodimeric partner of FXR and increased bile concentrations were found in patients with chronic liver diseases and HCC, it can be speculated that retinoic acid favours the formation of FXR/RXR heterodimer and bile acid excretion. Supporting this view, we demonstrated a dramatic reduction of cholestasis and hepato-necrosis parameters in patients treated with retinoic acid. Whether retinoic acid favours the formation of the FXR/RXR complex, however, in cirrhotic patients requires an appropriately designed study.

Table III. *The receptors expression for retinoids in the liver biopsies.*

N.	Initials	Sex	Histology	Survival (months)	RAR- $\alpha$	RAR- $\beta$	RAR- $\gamma$	RXR- $\alpha$
1	SE	M	HCC-T	10	+	+	+	+
2	PM	M	HCC-N	12	+	-	-	+
3	FM	M	HCC-N	24	+	+	+	+
4	CG	M	HCC-T	6	+	-	-	+
5	BA	M	HCC-N	26	+	-	-	+
6	TM	M	HCC-T	22	+	+	+	+
7	RM	M	HCC-N	10	+	-	-	-
8	BR	M	HCC-N	11	+	+	+	+
9	ML	M	HCC-N	18	+	-	-	-
10	TC	F	HCC-N	26	+	+	-	+
11	PD	F	HCC-N	24	+	-	-	-
12	FO	M	HCC-N	24	+	+	-	-
13	BF	M	HCC-N	41	+	-	+	+
14	BM	M	HCC-N	31	+	-	-	+
15	BA	M	HCC-N	16	+	+	+	+

Abbreviations: HCC-T, trabecular hepatocellular carcinoma; HCC-N, nodular hepatocellular carcinoma; RAR and RXR, retinoid acid receptors.

Although the effectiveness of tamoxifen alone in the treatment of patients with inoperable HCC has been reported to be inconsistent and inconclusive (29-31), in this pilot study we found the combination therapy of tamoxifen with retinoids to have a significantly greater effect. This finding could be due to a synergistic action between tamoxifen and retinoic acid in HCC patients, as has been shown previously in other patients with solid tumors (19).

Quality of life is a fundamental end point in oncological patient therapy (32). In our patients QoL was ameliorated: no aggressive pain therapy was required.

Our group's future direction is to study the retinoid resistance that is correlated with epigenetic changes of DNA, such as acetylation and methylation of transcriptional regions of the DNA, particularly concerning the retinoid receptor RAR- $\beta$  (33,34). Moreover, it is already well known that the combination of retinoids with histone deacetylase inhibitor (HDACIs) and demethylating agents have proved their ability to overcome the resistance to retinoids (35). For this reason, we are now performing our *in vitro* studies in hepatocarcinoma cell lines evaluating the efficacy of the association of HDACIs and retinoids.

In conclusion, despite the limitations of our pilot study regarding the small sample size, our data are sufficiently promising to propose that the combination of retinoic acid, tamoxifen and vitamin E may offer an alternative to tamoxifen alone as a safe treatment to ameliorate the survival and the QoL of patients with inoperable HCC. Our preliminary data warrant future controlled and randomised studies.

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