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Endolymphatic immunotherapy for advanced hepatocellular carcinoma: an update of our experience

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Abstract

Aim: We report an update of our experience on endolymphatic immunotherapy in patients with advanced hepatocellular carcinoma (HCC) not eligible for surgery.

Methods: From 2003 to 2009 we enrolled 39 patients with advanced HCC not suitable for surgery. Patients underwent monthly endolymphatic injections of 1.5×10^6-3.0×10^6 IL-2-activated peripheral autologous lymphocytes and 250U of IL-2. Blood biochemistry every 3 months and imaging studies every 6 months were performed. Evaluation of the results was done according to clinical and pathological characters mainly including etiology, Child-Pugh class, size and number of lesions, α-fetoprotein, lymphadenopathy, vascular invasion, Response Evaluation Criteria in Solid Tumours criteria for tumour burden, biochemical parameters and survival rates.

Results: Ten patients completed 12 therapy cycles, 6 received 6 infusions, 10 only 3-4 injection and 13 patients received less than 3 injections and where considered not suitable for evaluation. No clinically significant adverse reactions occurred. Imaging studies showed no significant decrease in tumour mass. Survival of treated patients was significantly higher with respect to control group ($P < 0.0001$). The 1-year survival was 0% in the control group *vs.* 50% in the treated group. In addition survival of patients who completed 12 therapy cycles appeared higher with respect to patients who underwent less than 6 cycles without reaching statistical significance due to

the small number of patients. All patients with 12 completed cycles showed an improvement of 9 parameters or more.

Conclusion: Endolymphatic administration of immunotherapy appeared safe, easy to perform and effective in terms of survival. This study should encourage future large scale studies in order to reach a firmer conclusion and define uniform inclusion criteria.

Keywords: Hepatocellular carcinoma, endolymphatic, immunotherapy, survival

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy globally and the third leading cause of malignancy - related mortality worldwide. The incidence of HCC is still higher in some African and Eastern Asian regions. This cancer represents 3%-6% of all solid tumours in the USA and Europe[1,2]. Hepatitis B virus (HBV)/hepatitis C virus (HCV) infection and alcohol abuse seem to be the main causes of the spread of HCC in Western countries[3]. Despite the established efficacy of screening programs for at-risk individuals, the diagnosis is usually performed at later stages of disease, wherein the tumour characteristics or liver disease progressions do not allow for curative therapeutic approach[4,5]. Many criteria have been proposed for the staging of HCC, combining different prognostic factors. The current treatment of HCC is based on the Barcelona Clinic Liver Cancer (BCLC) classification[6,7] including stages of the disease, macroscopic features of the lesion and liver function parameters as identified by Child-Pugh scoring system. Curative surgical treatment appears suitable in 30%-35% of all diagnosed cases[8,9], therefore much effort is directed towards new therapeutic agents. Encouraged by the good results obtained from treating metastatic renal cell carcinoma with immunotherapy[10], we offered the same procedure, with palliative intent, to patients with advanced disease who were not eligible either for hepatic resection or for percutaneous ablation based on BCLC classification obtaining interesting preliminary results[11] before approval of a new drug for treatment of HCC[12]. Sorafenib® is the only approved drug for patients with advanced HCC but has shown limited activity[13]. It acts as a multikinase inhibitor suppressing cell proliferation and angiogenesis. Recently it has been reported that other oncogenic targets may contribute to the anti-proliferative activity of the drug[14,15]. Herein we report the results of our pilot study in a cohort of patients with HCC in the pre-terminal stage who were not suitable for any curative interventions, before Sorafenib® - period.

METHODS

From January 2003 to March 2009, 39 patients with advanced HCC were enrolled in our study. Among these, 26 underwent at least 3 cycles of immunotherapy, but only 16 who completed at least 6 cycles were able to evaluate the efficacy of the treatment. In 13 patients the treatment was interrupted before the third cycle because of local skin reaction (n = 1), early death (n = 2) and worsened clinical conditions (n = 10). An historical control group is represented of 15 patients with similar characteristics of advanced HCC who underwent standard therapy without immunotherapy. The protocol of the immunotherapy which was already reported by our group[11] consisted in monthly endolymphatic infusions of 1.5×10^6-3.0×10^6 autologous activated lymphocytes (LAK) and 250IU of IL-2. Lymphocytes were obtained through the centrifugation of 30 mL of the patients' peripheral blood on a Ficoll-Hypaque gradient. The lymphocytes were then suspended in Roswell Park Memorial Institute-1640 (Sigma Aldrich, Germany) 2×10^6/mL and incubated with 20 U/mL of IL-2 at 37 °C for 72 h. After the incubation the cells were washed with saline solution and suspended in 5-10 mL of saline solution containing 250IU of IL-2.

Surgical procedure consisted of three steps. Firstly, the lymphatic vessels on the back of the foot was identified using the standard lymphographic technique (subcutaneous injection of violet patent blue between two finger). Then the main lymphatic was isolated and cannulated with a needle catheter (27G). A syringe containing the

cells suspended in 5 saline mL with 250U of IL-2 was connected to a pump for micro-injections (0.5 mL/min): the infusion lasted 10-20 min. The patients were also i.m. administered with chlorphenamine maleate (GSK, Brantford, UK) and ranitidine (GSK, Brantford, UK) 1 h before the treatment, in order to block H1 and H2 lymphocytes receptors and reduce possible side effects.

For evaluating the impact of the treatment on the tumour mass we adopted Response Evaluation Criteria in Solid Tumours criteria, a well known simple and pragmatic methodology to evaluate the activity and efficacy of therapies towards tumours[16]. In addition every three months we evaluated 12 biochemical parameters on the peripheral venous blood of the patients, i.e., alanine-amino-transferase (ALT), aspartate-amino-transferase, gamma-glutamil-transferase (GGT), bilirubin (BIL), alkaline phosphatase (ALP), α-fetoprotein (AFP), platelets, white blood cells, total plasmatic proteins, albumin, prothrombin time, creatinine (Cr). The minimal acceptable response to the therapy was defined as an improvement of at least 7 of these biochemical parameters. Finally we compared the survival rate of the treated patient to that of the non-treated patients (control group).

The present study was conducted in accordance with the ethical standards of the Helsinki Declaration (1964, amended most recently in 2008) of the World Medical Association. The local Institutional Review Board approved the use of the database for this retrospective review of the case files. Each patient provided written consent, and all patient information, including illustrations, were anonymous.

Statistical analysis

Data are represented as mean (range) for continuous variables and as *n* (%) for categorical variables. The χ^2 test or Fisher's test and the Student's *t* test were used to analyse categorical and continuous variables.

Survival analysis was performed using the Kaplan-Meier method and the log-rank test. *P*-values < 0.05 were considered significant. Data were analysed using SPSS (version 15.0) (SPSS Inc., Chicago, IL, USA).

RESULTS

In Table 1 are reported and compared the main clinical and pathological characteristics of treated patients and control group. There were no statistically significant differences between the two groups. Among the 26 patients enrolled in our study, ten patients completed 12 therapy cycles, six received 6 infusions and ten patients underwent only 3 or 4 procedures.

Twelve patients showed a partial response to the therapy that is amelioration of at least 7 out of 12 biochemical parameters considered [Figure 1]. Moreover, all the patients who completed the 12 cycles showed an improvement in 9 or more of the analysed parameters [Figure 1].

All parameters, but ALP and GGT, either improved or remained stable in more than 50% of the cases [Figure 2].

The regression of the neoplastic mass was not evident at the imaging studies in neither group, but in the treated group we observed 34% of patients with stability after 12 cycles and 0% of stability in the other patients treated with ≤ 6 cycles of immunotherapy.

The survival rate was measured from the beginning of the therapy, and analyzed with the Kaplan-Meier curve. The difference between the treated group and the control group was calculated with log-rank test and found to be statistically significant ($P < 0.0001$). The 1-year survival was 0% in the control group *vs.* 50% in the treated group [Figure 3].

A striking difference (even though not statistically significant due to small numbers of the groups) can be noted between the group of patients who completed the 12 cycles and those with < 6 cycles; 1-year survival was

Table 1. Clinical and pathological characteristics of patients with advanced hepatocellular carcinoma

Parameter	Treated group (*n* = 26)	Control group (*n* = 15)	*P*
Age	Mean 69 years (49-76)	Mean 67 years (52-75)	0.648
> 60 years	20 (77%)	11 (73%)	
< 60 years	6 (23%)	4 (27%)	
Gender			0.693
Male	22 (85%)	12 (80%)	
Female	4 (15%)	3 (20%)	
Etiology			0.972
HCV	5 (19%)	3 (20%)	
HBV	7 (27%)	5 (33%)	
HCV + HBV	4 (15%)	2 (14%)	
Other	10 (39%)	5 (33%)	
Liver			1.000
No cirrhosis	8 (31%)	4 (27%)	
Cirrhosis	18 (69%)	11 (73%)	
Child-Pugh			0.992
A	19 (73%)	11 (73%)	
B	5 (19%)	3 (20%)	
C	2 (8%)	1 (7%)	
Ascites			0.730
Yes	7 (27%)	5 (33%)	
No	19 (73%)	11(77%)	
Splenomegaly			0.512
Yes	16 (61, 5%)	11 (77%)	
No	10 (38, 5%)	4 (23%)	
α-fetoprotein			1.000
< 200 ng/mL	13 (50%)	8 (53%)	
> 200 ng/mL	13 (50%)	7 (47%)	
N. of HCC lesions			1.000
Single	2 (8%)	1 (7%)	
Multiple	24 (92%)	14 (93%)	
Tumor size			0.644
Single nodule	6.3 cm × 5.5 cm; 7 cm × 5.5 cm	7cm × 6.5 cm	
Multiple nodules (median, range)	5 cm (2-9 cm)	5 cm (1-8 cm)	
Lymph node positive			1.000
Yes	5 (19%)	3 (20%)	
No	21 (81%)	12 (80%)	
TACE			1.000
Yes	8 (31%)	4 (27%)	
No	18 (69%)	11(73%)	
Previous liver resection			1.000
Yes	17 (65%)	10 (67%)	
No	9 (35%)	5 (33%)	
Portal vein infiltration e/or thrombi			1.000
Yes	8 (31%)	4 (27%)	
No	18 (69%)	11 (73%)	
Caval thrombi			1.000
Yes	2 (8%)	1 (7%)	
No	24 (92%)	14 (93%)	

HBV: Hepatitis B virus; HCV: Hepatitis C virus; TACE: transarterial chemoembolization

100% in the group that completed 12 cycles *vs.* 20% in patients with < 6 cycles of therapy [Figure 4].

We compared the characteristics of patients on the basis of the therapy cycles (12 cycles or < 6 cycles) and observed that all ten patients who completed the 12 cycles were Child A and without vascular infiltration of portal vein and seven of them had a value of AFP < 200 ng/mL. However, we have to remark that hepatic reserve and tumor burden of HCC could be affecting the survival of the patients.

Among the remaining 16 patients (group ≤ 6 cycles), 11 were Child B and C, 8 showed vascular infiltration, 10 had a value of AFP > 200 ng/mL and 1 patient had bone metastases. These factors (Child B or C, AFP > 200 ng/mL, portal infiltration and the presence of extrahepatic malignancy) may be considered as

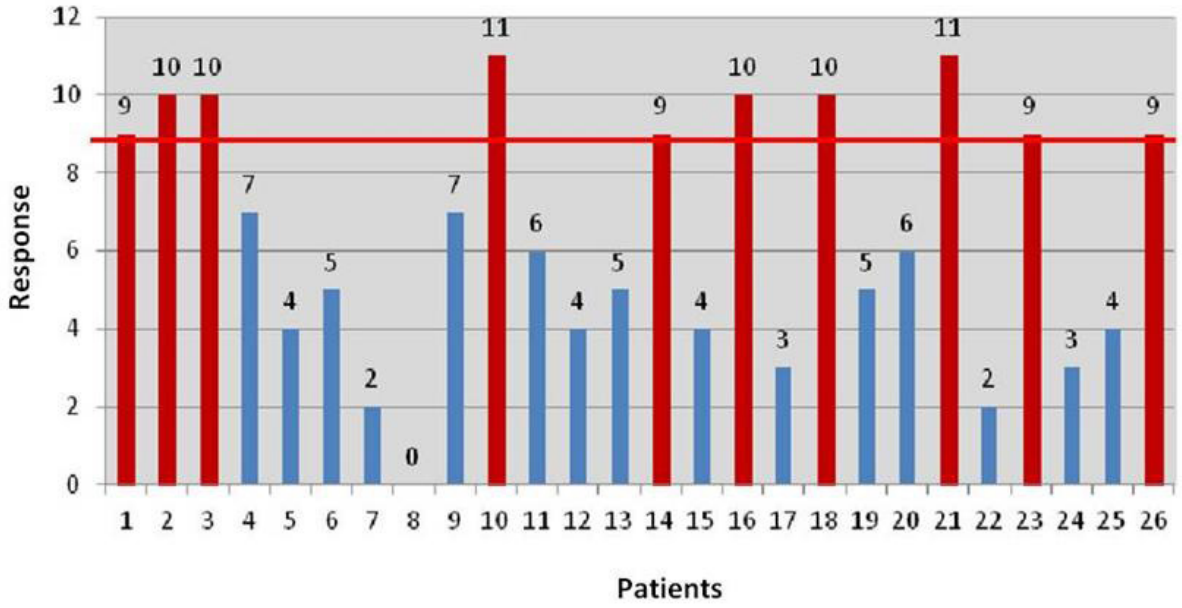

Figure 1. Trend of biochemical parameters in treated patients with endolymphatic immunotherapy according with number of therapy cycles. Patients who completed 12 therapy cycles (red color) *vs*. patients with less than 6 cycles (blue colour)

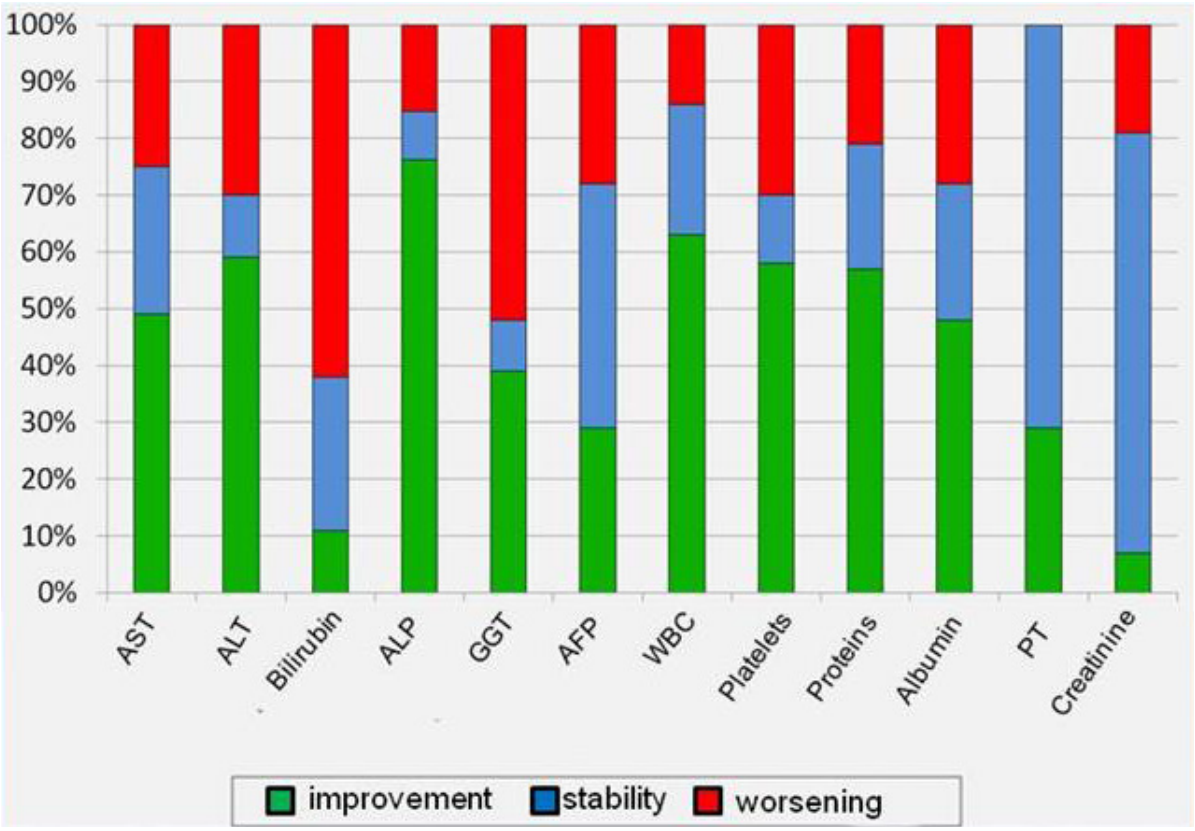

Figure 2. Percentage of improvement (green), stability (blue) o worsening (red) of biochemical parameters. AST: aspartate-amino-transferase; ALT: alanine-amino-transferase; ALP: alkaline phosphatase; GGT: gamma-glutamil-transferase; AFP: α-fetoprotein; WBC: white blood cells; PT: prothrombin time

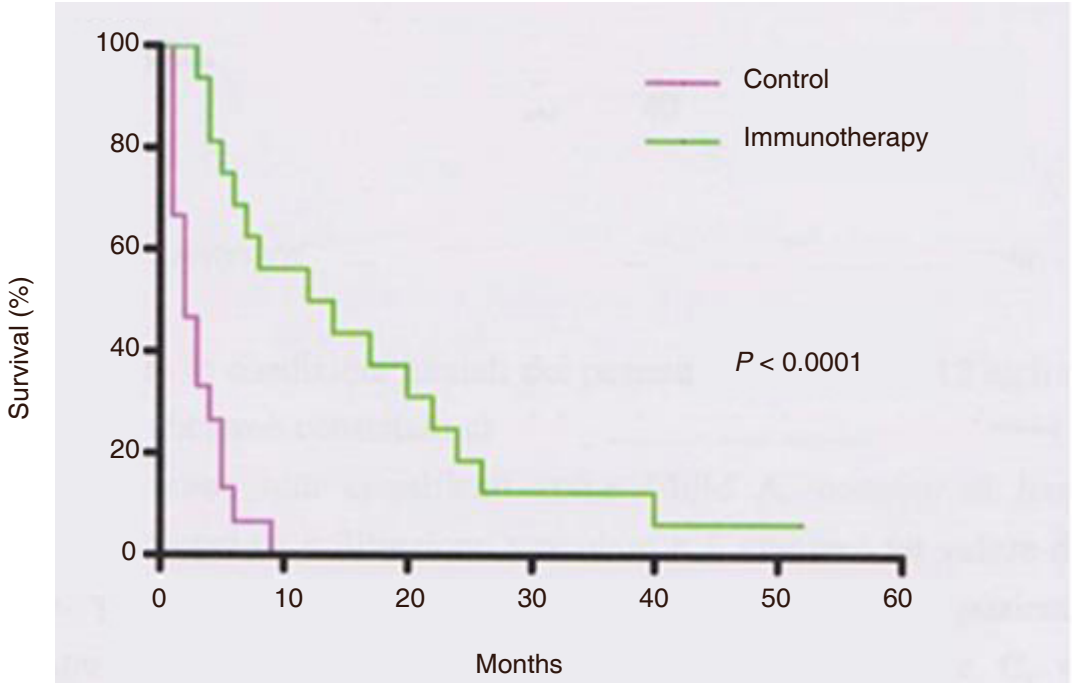

Figure 3. Kaplan-Meier survival analysis of patients with advanced hepatocellular carcinoma (endolympahtic immunotherapy *vs*. control group) ($P < 0.0001$)

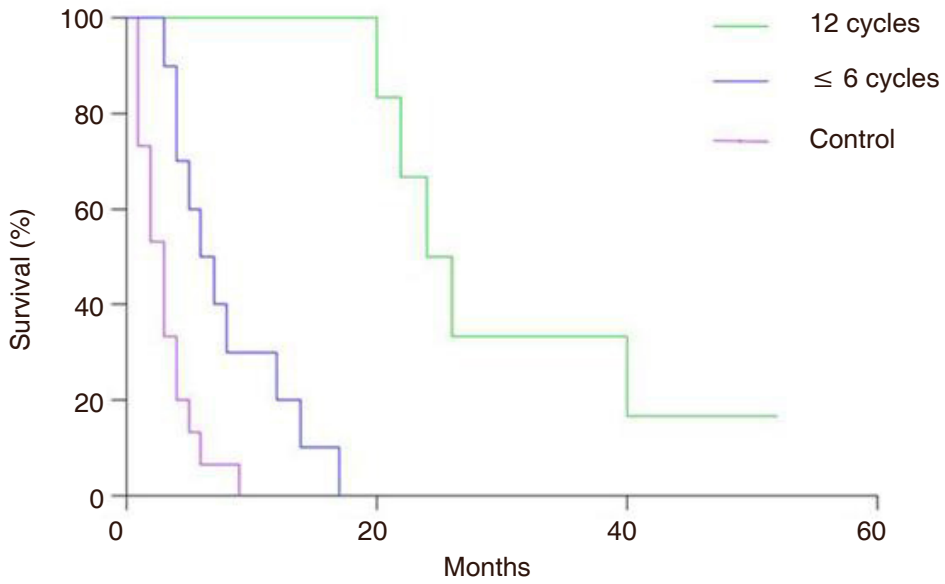

Figure 4. Kaplan-Meier survival analysis of patients with advanced hepatocellular carcinoma according to cycles of immunotherapy therapy and compared to control group (P = ns)

poor prognostic factors but are necessary larger studies to define the exclusion criteria of the patients for endolymphatic immunotherapy.

DISCUSSION

HCC is a complex and heterogeneous tumor with multiple genetic aberrations. Several molecular pathways involved in the regulation of proliferation and cell death are implicated in the hepatocarcinogenesis in

addition to major etiological factors, i.e., HBV and HCV virus infections. Continuous oxidative stress also due environmental factors or cellular mitochondrial dysfunction, have recently been associated with hepatocarcinogenesis[17,18]. At present time Sorafenib®, a multikinase inhibitor, represents the most promising therapeutic agent which has undergone extensive investigation up to phase III clinical trials in patients with advanced HCC. The combination with other target-based agents[14,15] could potentiate the clinical benefits obtained by Sorafenib®. Recently it has been reported that fasting had synergized with Sorafenib® in hampering HCC cell growth and glucose uptake[19]. Moreover, fasting could appear to normalize the expression levels of genes which are commonly altered by Sorafenib® in HCC cells. Thus, fasting or fasting-mimicking diet should be evaluated in preclinical studies for potentiating the activity of Sorafenib® in clinical use.

HCC patients are frequently cirrhotic with an associated deficiency of liver function that increases the toxicity of conventional chemotherapy, so immunotherapy could be considered a promising treatment option. Recent papers reporting clinical trials on immunotherapy for patients with advanced HCC mainly outlined the safety and feasibility of such therapeutic approach although the results were inconstant and not comparable[20,21]. The clinical results obtained by Onishi *et al.*[20] are very close to our own. Ten patients with HCC, three of whom had pulmonary metastasis, were treated with adoptive immunotherapy using autologous LAK cells plus recombinant IL-2. Patients received 15 μg per day of recombinant IL-2 consecutively (for 14 to 64 days), from day 7 prior to the first leukapheresis, and received 109 to 1010 LAK cells once or twice per week intravenously; the LAK cells had been generated from mononuclear cells obtained through leukapheresis. Previous administration of recombinant IL-2 prior to the first leukapheresis resulted in a remarkable increase of LAK activity in seven of nine cases in whom LAK activity had been poorly inducible even at high concentrations of recombinant IL-2. At the end of the treatment, liver tumor regression (34% and 63%, respectively, of two-dimensional size) was observed in two of two patients with a solitary tumor; no increase of liver tumor size was observed in seven patients with massive or multiple tumors, and no changes in the size or number of pulmonary metastatic tumors in any patients were observed. A decrease of more than 35% in serum α-fetoprotein level was noted in four of nine α-fetoprotein-positive patients. However, child's grades, performance status and LAK activity on entry into the study could not be used as parameters to predict therapy responsiveness. Neither serious side effects, significant changes of serum BIL, ALT nore Cr were noted. Thus, this treatment seems to be well tolerated even in advanced HCC with poor liver function reserve, and tumour regression could be expected in small-burden HCC.

In our study we aimed to demonstrate the efficacy of immunotherapy administered by means of endolymphatic injections while in the literature few studies on advanced HCC treated with different procedures[20,21] are available. In this first phase of our study we evaluated the safety and efficacy of the endolymphatic infusions of LAK and of IL-2 alone. Despite the small number of patients enrolled, the results obtained seems encouraging in terms of survival rate and improvement of biochemical parameters. We calculated the survival rate of the treated patient compared to historical control group of 15 patients with similar characteristics of advanced HCC who were not treated with endolymphatic immunotherapy (control group). The 1-year survival was 0% in the control group *vs.* 50% in the treated group.

Moreover concerning the survival a striking but not significant difference was observed between the group of patients who completed the 12 cycles and those who did not; 1-year survival was 100% in the group that completed 12 cycles *vs.* 20% in patients with that did not complete 12 cycles of therapy ($\leq$ 6 cycles). The immunological basis for the clinical effect on survival, mainly the changes in circulating lymphocytes, was not investigated yet. We observed that patients who underwent 12 cycles had no signs of vascular infiltration, levels of AFP lower than 200 ng/mL, no metastases and a Child-Pugh score of A. Since hepatic reserve and tumour burden of HCC could be the critical factors affecting the survival of the patients, further investigation in a large population of patients is mandatory. However, this analysis may allow us to consider these features as parameters for inclusion in future studies as this category of HCC patients may have the largest benefit from

endolymphatic immunotherapy as a palliative strategy. The regression of the neoplastic mass, however, was not evident at the imaging studies in neither group. The low dosage of IL-2 is responsible for two other important advantages of this treatment: the virtual absence of major side effects and the low costs of the treatment. In conclusion we firmly consider immunotherapy a good prospective for the treatment of HCC both for its efficacy and for the low systemic toxicity in comparison to chemotherapy, which is often unacceptable in patients with a such compromised liver level. On the other hand, the detection of molecular factors predictive of response to anti-cancer agents such as Sorafenib® and the identification of mechanisms of resistance to anti-cancer agents[22] may probably represent another direction to improve the treatment of HCC.

DECLARATIONS

Authors' contributions

Concept and design, data acquisition, data analysis, manuscript preparation: Lugaresi M, Katz Y, Bertelli R, Ruhrman N, Puviani L, Cavallari G, De Vinci C, Pizza G, Nardo B
Critical revision and finalizing of the manuscript: Lugaresi M, Pizza G, Nardo B

Availability of data and materials

The data were strictly obtained from medical records according to the privacy policy and ethics code of our institute.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

The local Institutional Review Board approved the use of the database for this retrospective review of the case files.

Consent for publication

Consents from all of the patients were established prior to submission and all records were confidential.

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