



Original Research

## Effect of hydatid cyst antigens on inhibition of melanoma cancer growth in mouse model

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**Abstract:** Cancer is the main cause of death in the developed countries. There are some scientific evidences indicating that parasitic infections induce antitumor activity against certain types of cancers. Hydatid cyst is the larval stage of *Echinococcus granulosus*, which causes hydatidosis in human and livestock. We have already shown that vaccination of mice with hydatid cyst crude antigens and subsequently challenge them with cancer cells, causes inhibition of melanoma cancer growth. In this study, therapeutic effects of hydatid cyst antigens on C57/black mice that had already been challenged with melanoma tumor were investigated. In this experimental study, 6 groups of C57 black mice were subcutaneously inoculated with melanoma cancer cells (line B16F10) in PBS inside their chest site. After 2 weeks case groups were injected with hydatid cyst fluid, a fraction of cyst fluid, live protoscolices or BCG. control groups were injected with alum alone and other control group was left intact without any intervention. The size of each tumor was measured in all mice. Blood samples were also taken to estimate Interleukin-2 (IL-2), Tumor necrosis factor alpha (TNF- $\alpha$ ), Interferon gamma (IFN- $\gamma$ ) and Interleukin-4 (IL-4) levels. Treatment of mice bearing melanoma cancer with hydatid cyst antigens resulted in inhibition of tumor growth and the difference between mean size of tumor in case and control groups was statistically significant. Also, according to our results mean level of measured cytokines between case and control groups was statistically different. Hydatid cyst antigens have anti-melanoma activities and this effect may be related to immune response to parasite antigens.

**Key words:** Melanoma; *Echinococcus granulosus*; Hydatid fluid antigen; Cytokine; IL-2; IL-4; TNF- $\alpha$ ; IFN- $\gamma$ .

### Introduction

*Echinococcus granulosus* is a tapeworm parasite lives in intestinal tract of carnivores such as dogs. The larval stage of the worm, hydatid cyst, develops in various tissues, including liver, lung and brain of grazing animals as intermediate hosts (1). Human is an accidental host of the parasite and infected via ingestion of the worm ova which result in development of hydatid cyst in his tissues. Hydatid cyst outwardly consists of laminated layer, germinal layer and broad capsule containing protoscolices. The cyst is filled with a transparent and sterile liquid which is called hydatid fluid (2). Hydatid cyst fluid contains different antigens such as Antigen B, Antigen A and 78KDa fraction (3-5).

More recently, the hygiene hypothesis has been restated to explain the association between microorganisms and cancers(6). The ability of various infective agents to suppress cancer growth has been well documented both in humans and in experimental animal models (7, 8). Anti-cancer effects of hydatid cyst have also been shown in different investigations (9-12). Antigenic similarities between *E. granulosus* and some tumors types have been shown. As an example cancer-associated mucin-type O-glycan antigens are expressed by some helminth parasites (13). Some of these share antigens

of *E.granulosus* could be involved in the induction of a cross-reactive immune response which could be effective in inhibition of cancer growth (5).

Due to its capacity for metastasis and refusal to therapy melanoma is one of the most hazardous cancers (14). So many investigations have been performed to find out an effective immunotherapy method for treatment of metastatic melanoma(15).

Cytokines have been widely studied as potential therapeutic agents against tumors (16-18). They also have a role in regulation of immune response to hydatid cyst, as an example the presence of Interleukin-2 (IL-2), Interferon- $\gamma$  (IFN- $\gamma$ ), Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), Interleukin-4(IL-4), Interleukin-5(IL-5), Interleukin-6(IL-6) and Interleukin-12 (IL-12) in the sera of animals with the primary or secondary hydatid cyst infection and also human patients is well documented (19, 20).

We have already shown that vaccination of mice with hydatid cyst crude antigens and subsequent challenge of them with melanoma cells resulted in inhibition of melanoma tumor growth(11) In order to provide results that can be translated for human applications, in this work therapeutic effect of various antigens of hydatid cyst on growth of developed melanoma cancer in mouse model has been investigated.

## Materials and Methods

### Parasite and antigen preparation

In this experimental study, *Echinococcus granulosus* hydatid cysts were collected from sheep or cattle from a slaughter house in Isfahan, Iran. Cyst fluid was aspirated and examined for presence of live protoscolices. Collected cyst fluids were then centrifuged and the supernatant kept at -20 as hydatid cyst fluid crude antigen. The sediment which was a pellet of accumulated protoscolices was given two washes with isotonic saline. The protoscolices immediately injected to experimental mice as live protoscolices.

### Purification of 78 kDa Fraction of hydatid cyst fluid (HCF)

Fractions protein of HCF was purified by gel filtration chromatography and loaded on to Hi-Load 16/600 Superdex 75 prep grade column. The column was washed with 150 ml buffer (PBS-buffer, pH:7.7) and flow rate of 1 ml/min at room temperature(21). The Bradford assay was used for protein quantification at 595 nm. The results of chromatography were analyzed on 12% SDS-PAGE. Samples were resuspended in 1× protein loading buffer (60 mM Tris-HCl, 2% SDS, Glycerol 10 %, 0.01 % bromophenol blue, 5mM DTT) and after electrophoresis gels were stained with silver stain (22). Protein level of the purified 78KDa fraction was also estimated. The purified 78kDa fraction was then kept in -20° C until use.

### BCG (Bacillus Calmette–Guérin)

Was purchased from Pasture Institute (Tehran, Iran) and used as antigen.

### Cell culture

Murine melanoma cell( line B16F10) and C57black mice, were purchased from the Pasture Institute (Tehran, Iran). Cells were then cultured in RPMI medium supplemented with 10% FBS at 37C temperature, 5% CO2 and 95% humidity as we published before (11). Tumor cell viability was assayed using trypan blue staining.

### Animal model work

This study was conducted on 6 groups of inbred C57/Black female 5 -7 weeks old mice (6 mice in each group). All mice were injected subcutaneously with 5×10<sup>5</sup> Melanoma cell line B16F10 in PBS in their chest site. After 4 days when tumor was visible in small size, the mice in case groups injected with hydatid cyst fluid and Alum (group1), the Fraction and Alum (group2), BCG (group 3), 500 protoscolices/ mouse (group4). Two control groups were injected with Alum alone and other control group was left intact without any intervention. After that, size of tumors was measured in two dimensions. The tumor area was then calculated for each mouse using the following equation(23).

$$\text{Tumor area (mm}^2\text{)} = \pi \times \frac{(\text{diameter1} + \text{diameter2})^2}{4}$$

Mouse groups were monitored every three days and their mortality was recorded. Ultimately, the survival rates of mice in case and control groups were compared. Survival of mice was followed for 60 days.

### Detection of cytokine production

One week after last antigen injection, blood samples were taken from all mice and their sera were prepared to measure cellular immune responses. Cytokines including IL-2, IL-4, IFN- $\gamma$  and TNF- $\alpha$  were measured using Enzyme-linked immunosorbent assay (ELISA, eBioscience, USA) to evaluate cellular immune response. The level of cytokines was reported as picogram/milliliter (pg/ml).

### ELISA analysis

ELISA method was performed according to the instruction of manufacturer. Briefly different antibodies (anti: IFN- $\gamma$ , IL-2, IL-4 and TNF- $\alpha$ ) were coated on 96 well multiplates overnight at 2-8°C. The plates were washed with wash Buffer containing 0.05% Tween 20 and blocked with xELISA/ELISPOT (1% BSA in TBS) for 1 h at room temperature. The plates were then incubated with the different serum sample of mice for 2 h at room temperature. After aspiration and washing, the detecting antibody were added to each well, incubated for 1 h at room temperature. The plates were then aspirated and washed and incubated with the avidin-HRP complex (Vector) at room temperature for 30 minutes. After aspiration and wash, the substrate solution was added to each well and incubated at room temperature for 15 minutes and after that stop solution was added. Optical densities (OD) of wells were measured (at 405 nm) in a spectrophotometer (Epson) and the amount of each cytokine in samples were calculated according to the instructions of each kit.

### Statistical Analysis.

Kruskal-Wallis 1-way ANOVA (K sample) was used to compare data from various experimental groups.

P value <0.05 was considered statistically significant. Survival time was evaluated from the day of treatment until euthanasia, and the Kaplan-Meier test was used to compare mice survival time among the groups. All results were presented as means  $\pm$  SD. Data were processed using the IBM SPSS Statistics 20.0 software.

### Results

In the first experiment, 6 groups of mice were injected with melanoma cancer cells. After 4 days, groups 1 - 4 were treated with living protoscolices, hydatid cyst fluid, 78kDa fraction of hydatid cyst fluid and BCG. Groups 5 were injected with alum and group 6 was left intact as control groups. All hydatid cyst antigens including hydatid cyst fluid, the fraction, live protoscolices and also BCG significantly inhibited the growth of tumors in comparison with control groups. Results of tumor size of the above mice have been summarized in Table -1.

Life spans of the mice in all above groups were also estimated. The results revealed that in hydatid cyst fluid, the fraction, protoscolices and BCG injected mice, life span was significantly longer than that of control groups (Table- 2).

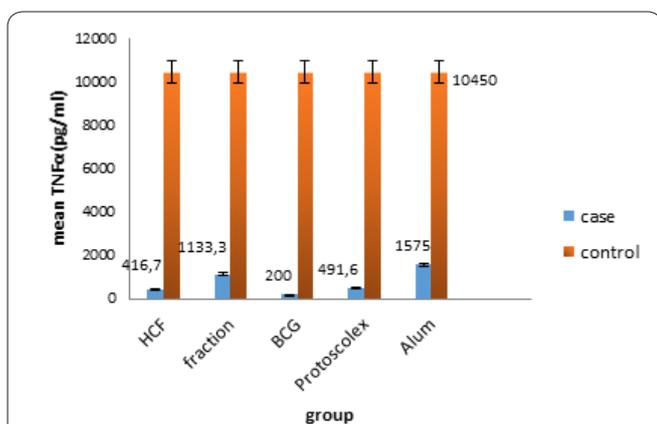
In all mice the serum level of IL-4, IL-2, IFN- $\gamma$  and TNF- $\alpha$  cytokines were estimated. The result showed that in mice that treated with hydatid cyst fluid (HCF), the fraction, live protoscolices or BCG (case groups)

**Table 1.** Comparison of life span of melanoma bearing mice treated with hydatid cyst fluid (HCF), a fraction of HCF, live protoscolices, BCG or alum alone with that of control groups (melanoma bearing mice without any intervention).

Antigen	Groups	N	Area 1		Area 2	
			Mean±st	P	Mean±st	P
HCF	Case	6	485.78±524.97	0.03*	654.6±543.3	0.00*
	Control	6	1135.63±419.27		1749.5±635.28	
Fraction	Case	6	524.24±507.71	0.04*	915±700	0.17
	Control	6	1124.77±419.27		1448.06±635.2	
BCG	Case	6	333.1±425.81	0.01*	618.44±349.41	0.03*
	Control	6	1124.77±419.27		1448.06±635	
Protoscolice	Case	6	733.58±244	0.09	797.29±494	0.01*
	Control	6	1135.63±426.11		1749.50±358.23	
Alum	Case	6	1363.28±53.71	0.80	1824.20±491.29	0.87
	Control	6	1135.63±426.11		1749.50±358.21	

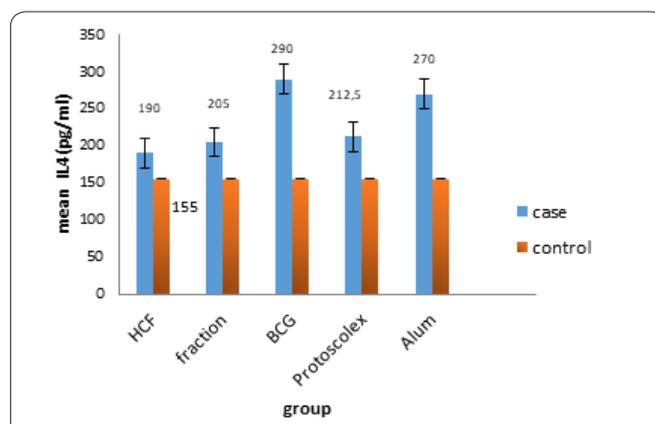
**Table 2.** Comparison of life span of melanoma bearing mice treated with hydatid cyst fluid (HCF), a fraction of HCF, live protoscolices, BCG or alum alone with that of control groups (melanoma bearing mice without any intervention).

Antigen	Groups	N	Life span	
			Mean±st	P
HCF	Case	6	21.33±4.03	0.01*
	Control	6	15.50±1.64	
Fraction	Case	6	20.67±4.41	0.02*
	Control	6	15.50±1.64	
BCG	Case	6	21.67±4.84	0.02*
	Control	6	15.50±1.64	
protoscolice	Case	6	15.83±4.16	0.85
	Control	6	11.83±2.9	
Alum	Case	6	13.67±0.51	0.17
	Control	6	11.83±2.9	



**Figure 1.** level of TNF-α in melanoma bearing mice treated with hydatid cyst fluid (HCF), a fraction of HCF, live protoscolices, BCG or alum alone with that of control groups (melanoma bearing mice without any intervention).

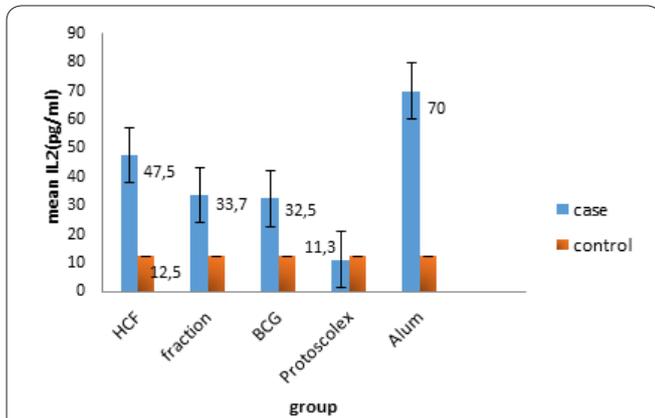
the level of TNF-α was significantly lower than that of control group. The level of IL-4 in all mice in case groups was significantly higher than that of control group. Also, the level of IL-2 in case groups treated with hydatid cyst fluid (HCF), the fraction or BCG was significantly higher than that of control group. Finally, the level of IFN-γ in mice treated with HCF, live protoscolices or BCG and fraction was significantly higher than that of control group. The results of level of different cytokines in case and control mice have been shown in figures 1-4.



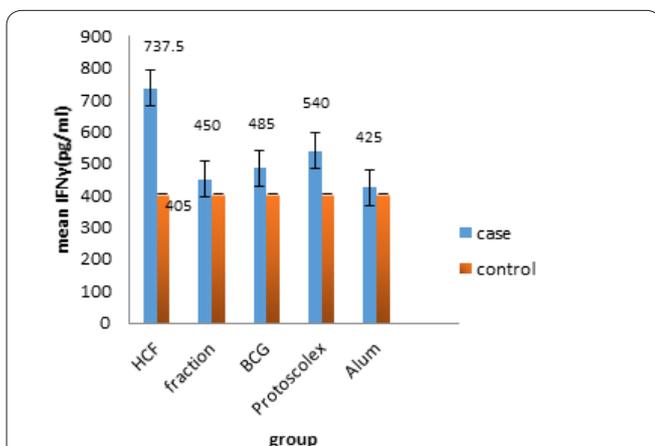
**Figure 2.** level of IL-4 in melanoma bearing mice treated with hydatid cyst fluid (HCF), a fraction of HCF, live protoscolices, BCG or alum alone with that of control groups (melanoma bearing mice without any intervention).

### Discussion

In this investigation, it has been shown that injection of different hydatid cyst antigens (including HCF, the fraction, live Protoscolices), and BCG to mice with melanoma cancer, results in reduction of tumor size in antigen injected mice. In agreement with our results, there are scientific evidences indicating that some parasitic and microbial infections interfere with tumor growth and have anticancer activities(11, 18, 24-27). Our results also showed that life span of mice injected



**Figure 3.** level of IL-2 in melanoma bearing mice treated with hydatid cyst fluid (HCF), a fraction of HCF, live protoscolices, BCG or alum alone with that of control groups (melanoma bearing mice without any intervention).



**Figure 4.** level of IFN- $\gamma$  in melanoma bearing mice treated with hydatid cyst fluid (HCF), a fraction of HCF, live protoscolices, BCG or alum alone with that of control groups (melanoma bearing mice without any intervention).

with HCF, BCG and the fraction was more than those of control groups and this difference was statistically significant ( $P < 0.05$ ). In agreement with our results it has been shown that life span of patients with leukemia who simultaneously were infected with *Strongyloides stercoralis* was higher than life span of leukemia patients without *Strongyloides stercoralis* infection (28). Also Malaria parasites increase the life span of mice with murine Lewis lung cancer (8).

The mechanism of anti-cancer effects of hydatid cyst antigens is not clear. It is possible that immune response raised against this parasite antigens, non-specifically interact with the cancer antigens and interfere with cancer growth. In this regard presence of common antigens between cancers and parasites has been demonstrated in different investigations (13, 29, 30). However, the ability of parasite antigens in inhibition of cancer cells growth in culture medium is not in agreement with the above conception. As an example, it has been shown that hydatid cyst Protoscolices induce death in fibrosarcoma cells and inhibit the proliferation of baby hamster kidney fibroblasts in vitro (12). Other possible mechanism of anti-cancer effects of hydatid cyst may be related to toxic effects of these antigens on cancer cells. This speculation is in agreement with anti-cancer effect of hydatid cyst antigens in culture medium (12). We for

the first time reported that a fraction (78KDa) of hydatid cyst fluid had anti-cancer activities. This fraction can be a candidate for cancer immunotherapy in human.

Cytokines have been widely studied as potential therapeutic agents to regulate the immune response against tumor cells (16). The result of the present study showed an increase in Th1-type cytokines (IL-2, IFN- $\gamma$ ) and Th2-type cytokines (IL-4) in melanoma bearing mice that were treated with hydatid cyst antigens. According to our results HCF antigens reduce TNF- $\alpha$  in all melanoma bearing mice. In melanoma cancer, it has been shown that TNF- $\alpha$  can induce cell invasion (31) and angiogenic. Also, it is shown that this cytokine up regulates malignant melanoma invasion and migration in vitro (31). So, it can be concluded that the hydatid cyst antigens decrease TNF- $\alpha$  which is in favor of tumor control.

Recent progress in understanding the role of IL-2 in regulating lymphocytes has led to new approaches in cancer immunotherapy (17, 18). Treatment with this cytokine has been approved for metastatic renal cell carcinoma and metastatic melanoma (16). Our data shows that treatment of melanoma bearing mice with hydatid cyst antigens increase IL-2 level (22). IL-4 receptors are expressed on a variety of human solid tumors. IL-4 alone or in combination with IFN-gamma may play a role in host immune response against some cancers (32). In this regard anti-tumor effect of IL-4 has been shown in murine experiments (33). Our data shows that treatment of melanoma bearing mice with hydatid cyst antigens increase IL-4 level and this increase was statistically significant ( $P \leq 0.05$ ).

Interferon- $\gamma$  (IFN- $\gamma$ ) is another cytokine with antitumor effects (34-37). Our Results showed that treatment of melanoma bearing mice with hydatid cyst antigens increase IFN- $\gamma$  level especially in HCF treated group and this difference was statistically significant ( $P \leq 0.05$ ). According to our results about the level of cytokines, it can be concluded that they may be involved in combating tumor growth and so may be considered as a possible mechanism for anti-cancer effects of hydatid cyst.

Results of this work indicated that treatment of melanoma bearing mice with antigens of hydatid cyst especially hydatid cyst fluid and its 78 KDa fraction resulted in significant inhibition of the cancer growth. So, this fraction may be a candidate for performance a clinical trial research in patients with melanoma cancer.

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### Interest conflict

None of authors has conflict of interest.

### Author's contribution

SRR did most of the lab works and prepared the first draft of the manuscript. MRF helped with performance of chromatography, AA was immunological consultant, AE helped with preparation of proposal work and correction of the manuscript, SD contributed to performance of lab works, HYD was the PI of this work and contributed to design of the work supervise of lab works and final correction of the manuscript.

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