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Original Article

Anticancer Activity of Hydatid Cyst Fluid along with Antigen B on Tumors Induced by 4T1 Breast Cancer Cell in a BALB/c Mice Model

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Abstract

Background: Studies on experimental model of cancer showed that hydatid cyst fluid (HCF) has antitumor activity. We aimed to investigate the effect of HCF and Antigen B (AgB) on 4T1 breast tumor cells in BALB/c mice.

Methods: This study was carried out in the Department of Parasitology, Tarbiat Modares University, Tehran, Iran from 2019 to 2020. There were two control groups of BALB/c mice (one group were injected with aluminum sulfate and another group with PBS), and six groups, injected via the intraperitoneal route with 100, 300 and 500 µg/ml concentrations of HCF, AgB diluted in 100 µl PBS, and alum. Seven days after the last treatment, 7×10^5 4T1 cells were subcutaneously injected into the right flank of BALB/c mice.

Results: The difference between the mean size of the tumor in the case and control groups was statistically significant ($P<0.05$). The mean level of cytokines between the case and control groups was statistically significant ($P<0.05$). Our histopathological studies showed a reduction in both tumor volume and carcinogenesis in groups injected with 300 µg/ml HCF and 500 µg/ml AgB. The antitumor activity of HCF and AgB may be related to the immune responses against these antigens.

Conclusion: We suggest that polarization of the Th1/Th2 ratio toward the Th1 pathway occurred in groups injected with HCF and AgB. More comprehensive and precise experiments using different hydatid cyst components are required to investigate their prophylactic effects on breast cancer.

Introduction

Hydatid cyst caused by the larval stage of the dog tapeworm, *Echinococcus granulosus*, is a neglected zoonotic infection of public health importance in both developing and developed countries of the world (1,2). It is a unilocular bladder filled with hydatid cyst fluid (HCF) and it grows in different organs, particularly in the liver and lungs (3,4).

Breast cancer is among the most prevalent cancers of women in the world with a high mortality rate and is the second most common female malignancy (5). Public health approaches to breast cancer control have highlighted the important role of prophylactic measures and the need for practical, low-cost interventions with the least toxicity (6).

Among certain causes of cancer in humans, there are carcinogenic parasites such as *Schistosoma haematobium*, *Clonorchis sinensis* or *Opisthorchis viverrini*, which induce bladder cancer and cholangiocarcinoma, accordingly (7). Contrarily, the prophylactic effects of some parasitic infections on different malignancies have been demonstrated. The antitumor activity of several parasitic antigens such as *Toxoplasma gondii*, *Trypanosoma cruzi*, *Plasmodium yoelii*, and *Trichinella spiralis* is illustrated through experimental surveys (8,9).

Several antigenic components within HCF have modulatory effects on the host immune response to support the development and survival of the parasite and also stimulate the cellular and humoral immune system in the host's body (10–12). Epidemiological studies have shown that HCF has antitumor effects (13–15).

One of the other components of the hydatid cyst, Antigen B (AgB), is a 120–160 kDa oligomeric thermostable lipoprotein composed of three subunits at 8/12, 16, and 20 kDa (16). As a potential protease inhibitor, AgB plays a major role in the reduction of neutrophil recruitment. It has been proved that neutrophil (NE) populations in the tumor microenvironment are highly involved in angiogenesis, tumor growth initiation, progression and metastasis (17).

Some of the parasitic antigens induce apoptosis through modulating the immune responses against metastasis, angiogenesis and proliferative signals (9). The current study was performed to evaluate the antitumor activity of HCF and AgB on 4T1 breast cancer cells in BALB/c mice.

Materials and Methods

This study was conducted in the Department of Parasitology, Tarbiat Modares University, Tehran, Iran during February 2019-September 2020.

Ethical statement

The current study was approved by the Ethical Committee of the Faculty of Medical Sciences, Tarbiat Modares University (Approval ID: IR.MODARES.REC1398.79).

Preparation of Hydatid cyst fluid

Sheep hydatid cysts were obtained from abattoir of Guilan Province, Iran. Hydatid fluid was aspirated and collected under aseptic condition, centrifuged at 2000×g for 2 min at 4 °C and the supernatant was collected and dialyzed with 5 mM acetate buffer (pH 5) overnight at 4 ºC. The dialyzed fluid was centrifuged at 50,000 g for 30 minutes to remove the albumin. After that, the pellet was mixed with 0.2 M phosphate buffer (pH 8). Saturated ammonium sulfate was employed in order to remove immunoglobulins (18). A dialysis membrane (cut-off value 12,000) was used to concentrate the prepared sample. Thereafter, samples were pooled and protein concentra-

tion of the fluid was measured using Bradford assay (19).

Preparation of AgB

AgB was obtained using a boiling water bath for 15 min and centrifuging them at 50,000g for 60 min. Polyacrylamide gel electrophoresis with 12.5% sodium dodecyl sulfate (SDS-PAGE) and Coomassie brilliant blue staining was used to evaluate the presence and quality of AgB subunits based on protocols described by Laemmli using the BIO-RAD apparatus (18). Subunits at 8-12, 16, and 20-24 kDa are attributed to AgB of hydatid cyst (20).

Cell line, Animal model, Antigen injection, and implantation of tumor cells

Mouse breast adenocarcinoma (4T1) cell line was purchased from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran) and cultured in RPMI-1640 medium (Gibco: AC16-012) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco:42F0375K), 2mM L-glutamine, 100 µg/ml of streptomycin (Gibco, USA) and 100 units/ml of penicillin. The cells were kept in a humidified incubator at 37 °C in a 5% carbon dioxide (CO_2) condition. Subsequently, the cells were harvested with 0.5 g/l trypsin along with 0.2 g/l Ethylenediaminetetraacetic acid (EDTA) for 3min.

The experiment was performed on 80 female BALB/c mice (6-8 weeks old) purchased from Royan Institute for Stem Cells and Developmental Biology (Tehran, Iran).

Animals were randomly divided into eight groups, with 10 mice in each group. One of the control groups received adjuvant (aluminum hydroxide=alum) via intraperitoneal injection and the other was injected with PBS. As previously described, mice in the experimental groups were injected intraperitoneally (on days 1, 7, 15, and 23) with different concentrations (100, 300, and 500 µg/ml) of HCF and AgB diluted in 100 µl PBS and alum (14). Seven days after the last treatment, a subcutaneous injection into the right flank was performed with 7×10^5 4T1 cells in 100 µl PBS (Fig. 1). Then, mice were kept in a laboratory condition without any treatment.

Fig. 1: Schematic illustration of AgB and HCF injection and 4T1 implantation in BALB/c mice

Tumor volume measurement

To determine the prophylactic effect of HCF antigens and AgB on 4T1 breast tumor growth in BALB/c mice, 7 days after cancer cell inoculation, the animals in each group were inspected for tumor growth, and tumor size was measured on alternate days for up to 4 weeks using a digital caliper vernier (Mitutoyo, Japan), and the volumes were calculated using the following formula: $V_t = (\pi/6) \times LW^2$

(V_t = tumor volume, $\pi = 3.14$, L = length of tumor, and W = width of tumor).

Table 1 is provided to show the tumor size reported on days 15, 19, 23, 27, and 31. On day 31, the mice were humanely euthanized by CO_2 inhalation, without removing animals from their cages.

Table 1: The tumor volume (Mean ± SEM, mm^3) of mice in control groups and mice injected with different concentrations of alum-absorbed hydatid cyst fluid, and antigen B (100, 300, and 500 µg/ml) following determined days after challenge with 4T1 tumor cells

Experimental Groups	***Day after cell inoculation***				
	Day 15th	Day 19th	Day 23th	Day 27th	Day 31th
Mice injected with AgB					
Group 1 (100 µg/ml)	271,8 ± 22,58	457,4 ± 35,17	673,3 ± 81,43	841,8 ± 38,25	1011,6 ± 45,39
Group 2 (300 µg/ml)	232,6 ± 20,28	296,5 ± 31,64	373,2 ± 66,48	472,8 ± 30,25	552,1 ± 66,53
Group 3 (500 µg/ml)	256,8 ± 46,38	365,3 ± 46,19	423,6 ± 33,27	537,2 ± 50,5	690,6 ± 28,74
Mice injected with HCF					
Group 1 (100 µg/ml)	240,6 ± 23,59	423,9 ± 47,78	651,6 ± 34,85	811,3 ± 90,76	1134,1 ± 33,30
Group 2 (300 µg/ml)	168,9 ± 47,73	277,4 ± 55,38	354,5 ± 83,80	429,5 ± 91,51	501,4 ± 97,41
Group 3 (500 µg/ml)	241,8 ± 46,38	301,3 ± 84,16	414,6 ± 63,24	526,0 ± 160,5	604,0 ± 126,4
Control groups					
Control 1 (Alum)	290,2 ± 39,25	391,4 ± 64,91	665,8 ± 91,55	934,0 ± 113,8	1250,4 ± 233,4
Control 2 (PBS)	296,5 ± 34,87	387,8 ± 52,31	698,8 ± 84,55	912,0 ± 102,7	1292,0 ± 257,2
P value	NS	NS	NS	<0.05	<0.05

NS: Not significant

Measurement of cytokines in serum

Serum specimens were separated from the collected blood of mice (72 hours after the last exposure to the antigens). Serum levels of Th1 [IL-2, IL-12, tumor necrosis factor-alpha (TNF-α), interferon-γ (IFN-γ)] and Th2 [(IL-4 and IL-6)] cytokines were measured using mouse enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen), according to the manufacturer's protocol. The optical density (OD) was calculated at 450 nm using a microplate reader (BioTek Instruments, Winooski, VT, USA).

Histopathology

The mice were sacrificed on day 31, and the tumor mass was removed for histopathological evaluation. Briefly, the excised breast tumor tissue was fixed in 10% formaldehyde solution. The routine paraffin technique was applied to process the tissues. Then, the prepared sections (4 µm) were stained via hematoxylin and eosin (H & E) staining method, and histological identification was done by OLYMPUS-BX51 microscope (10×, 40×) (21).

Statistical analysis

Statistical analyses were performed via two-way analysis of variance (ANOVA) followed by a post–hoc Bonferroni's test to evaluate the significant differences among the groups. SPSS Statistics 22 software (IBM Corp., Armonk, NY, USA), Graph Pad Prism version 8.0.1, and Paired Student's *t*-test were employed to analyze differences in the experiments for statistical significance. The analysis of results was represented as means ±SD and 95% confidence interval (CI). Values of $P < 0.05$ were regarded as statistically significant.

Results

Tumor size

There was a significant increase in the tumor size of mice in control groups compared to the other groups ($P< 0.05$). In animals injected with HCF (300 µg/ml) and AgB (500 µg/ml), the tumor size was considerably smaller than in control groups (after 31 days). However, the lowest tumor size was observed in animals from HCF groups (Table 1).

Cytokine level determination

The level of Th1 cytokines (IL-2, IL-12, TNF-α, IFN-γ) was elevated in animals inject-

ed with HCF and AgB compared to the control groups ($P< 0.05$). In addition, the serum levels of Th2 cytokines (IL-4 and IL-6) were decreased in animals injected with HCF and AgB ($P < 0.05$). There was a significantly lower amount of Th2 cytokines in groups injected with 300 µg/ml of HCF. As well, the higher amounts of Th1 cytokines in these groups were significant ($P< 0.05$). Fig. 2 shows the serum concentration of cytokines in all groups 72 hours after the last exposure to antigen at different concentrations.

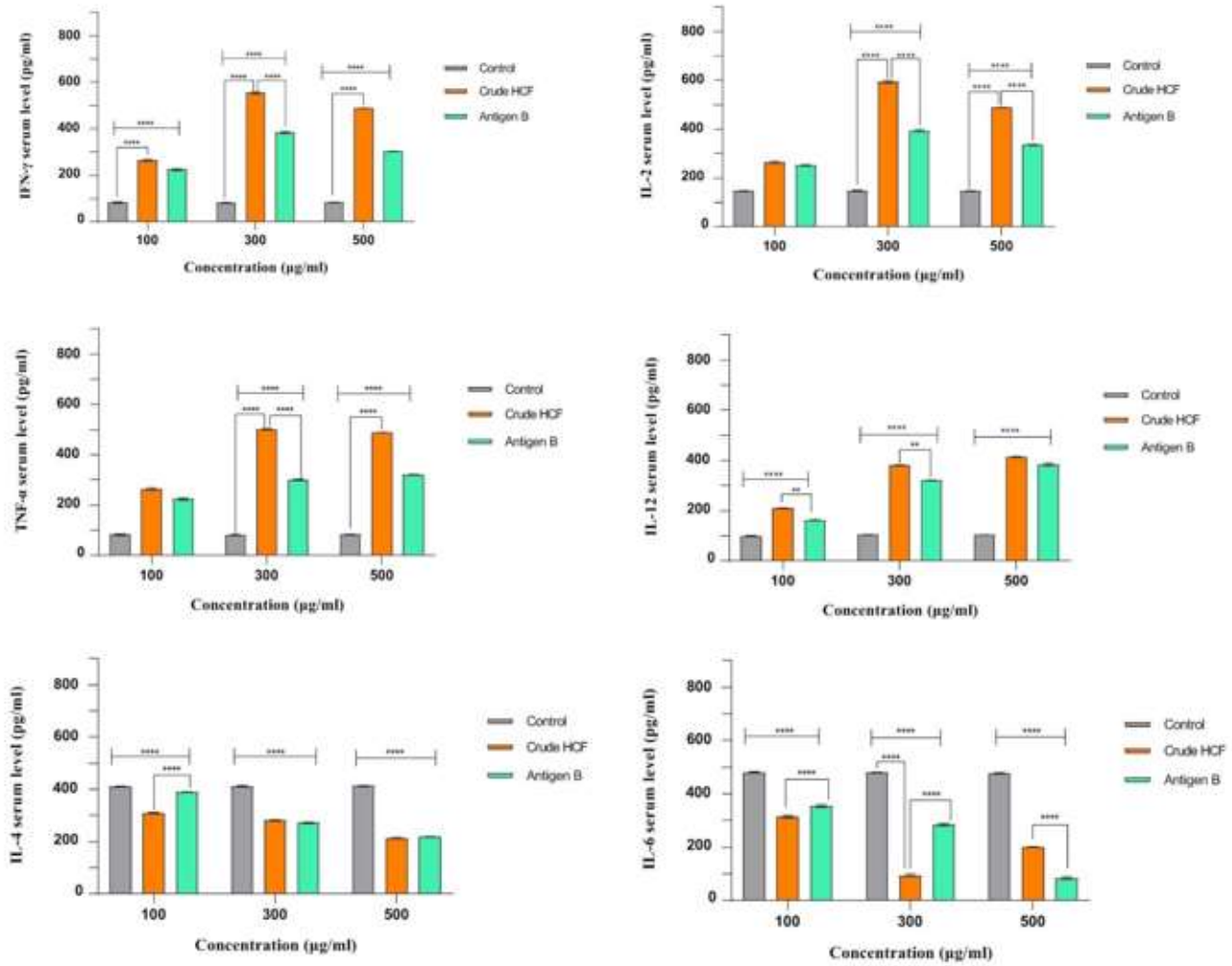

Fig. 2: The serum levels of Th1 (IL-2, IL-12, TNF-α, IFN-γ) and Th2 (IL-4 and IL-6) (pg/ml) cytokines in BALB/c mice 72 hours after the last exposure to different concentrations (100, 300 and 500 µg/ml) of AgB and HCF and in control groups. The results show mean ± SEM and asterisk indicates ($P < 0.05$)

Histopathological studies

The sections of breast tissue from the control group revealed infiltration of tumor cells and neoplastic cells with pleomorphic, large-shaped hyperchromatic nuclei and a small amount of cytoplasm (Fig. 3A). The tissue sections from AgB (100 µg/ml), HCF (100 µg/ml) and AgB (300 µg/ml) injected groups showed a histological pattern similar to the control group and there was an increased mitotic figure, eosinophilic cytoplasm, swelling of nuclei and vacuolation of cytoplasm (Fig. 3B, 3C and 3D). In HCF (300 µg/ml and 500 µg/ml) and AgB (500 µg/ml) groups, primary

breast tumors developed. However, granulation and/or fibrosis were seen with no mitotic figure and there was not an evident atypia. The rate of the morphologically normal cells was significantly higher than cells with nuclear pleomorphism (Fig. 3E, 3F, and 3G).

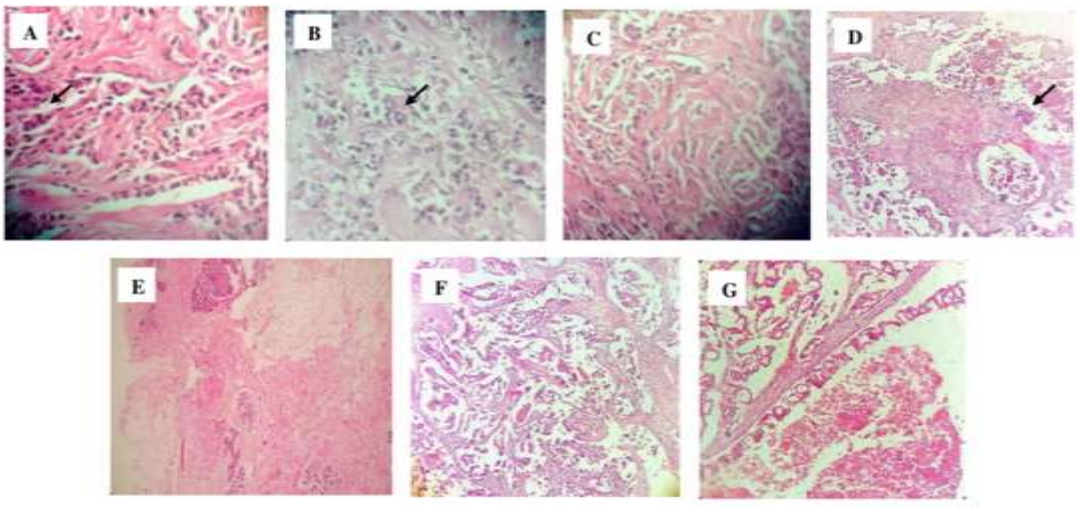

Fig. 3: Histological findings of breast cancer tissue (stained with H & E) induced by 4T1 cell line with a magnification of (×10) and (×40); (**A**) control group; (**B**) group injected with 100 µg/ml AgB and HCF; (**C**) group injected with 100 µg/ml HCF; (**D**) group injected with 300 µg/ml AgB; (**E**) group injected with 300 µg/ml HCF; (**F**) group injected with 500 µg/ml AgB; (**G**) group injected with 500 µg/ml HC

Discussion

Particular attention is paid to different antigenic components of helminthic parasites to be an applicable candidate for the prevention and treatment of cancers, autoimmune disorders, and inflammatory diseases, as they control the immune system through regulatory pathways (8,22,23). This study evaluated the antitumor activity of HCF and AgB on the 4T1 breast cancer cell line in BALB/c mice.

We outlined that tumor size was decreased in HCF and AgB groups compared to the control group. Nevertheless, the progression in tumor growth was lower in HCF groups than in AgB groups. In agreement with these results, previous studies have shown that HCF antigenic contents inhibit tumor growth in a mouse model of melanoma cancer (11,24). Likewise, a study showed the HCF-driven anticancer effect in the murine model of colon cancer (14). Moreover, the property of causing apoptosis by HCF and protoscolices has been described via in vitro studies before (12, 25–27). Karadayi et al. indicated that the serum samples of hydatid disease patients have a cytotoxic effect on human lung small cell carcinoma cells (28).

Th1 cytokines are involved in the upregulation of an effective anti-cancer immune response. While, Th2 cytokines are important in the upregulation of humoral immunity and appear to improve breast tumor progression (29). The increased levels of Th1 cytokines (IL-2, IL-12, TNF-α, and IFN-γ) in HCF and AgB groups can illustrate that T cells are shifted to type-I immune response.

IFN-γ plays an important role in cancer immune surveillance and inhibits tumor growth by directly affecting tumor cells (30,31). Antigen-presenting cells (APCs) are activated by IFNγ signaling to enhance IL-12 and IL-18 production (30,32). Also, increased amounts of IFN-γ inhibit the angiogenesis of the tumor

tissues (32). The present research was consistent with studies that have proven hydatid cyst-driven production of IFN-γ and the role of Th1 in regulation of hydatid cyst (16). As well, former investigations showed a measurable amount of IFN-γ in the serum of hydatid patients (33).

We observed a decrease in Th2 cytokines (IL-4 and IL-6) in AgB and HCF groups. Studies on preclinical subjects of cancer suggested that IL-6 is a promoter of tumorigenicity, angiogenesis, and metastasis (34). An experimental study on breast cancer showed that Hydatid Cyst Wall Antigens considerably increased the serum levels of IL2, TNF-α, IFN-γ, and IL4 in mice (35). Similarly, another research discovered that IL2, IFN-γ, and TNF-α serum levels were significantly increased in the group injected with HCF and 78 kDa fraction antigens (36). An investigation of serum samples in patients with colorectal cancer showed that it was associated with a decrease in IL-12 level and an increase in IL-10 level (37).

The components within HCF can activate dendritic cells to induce IL-12 and IL-6 production and stimulate a combined Th1/Th2 response against parasitic antigens (38). However, we found that the amount of IL-6 was reduced to the lowest level in the HCF and AgB groups (300 µg/ml), which antitumor functions were detected at the highest rate.

HCF contains a wide range of immunomodulatory elements affecting both cellular and humoral immune responses in the host's body (39). Moreover, the function of cellular immunity is considerably improved during the initial stages of hydatid cyst disease (40). During hydatidosis, the type and amount of antigens released by the parasite have a main role in the alternation of immune responses (41).

A review of experimental studies revealed some common antigens between parasites and autoimmune diseases such as Crohn's disease, ulcerative colitis, multiple sclerosis, and asthma (8,21,42). The antigenic similarities between some parasites and cancers cause to immune responses against parasitic infections, which can strengthen the immunogenicity of tumor antigens (43). There are some types of peptides existing in the larval and adult stages of *E. granulosus* as well as in the serum of hydatid patients that induce antitumor activity (44).

Th1 cells are essential to initiate an effective antitumor immune response and they can recognize mucin-like peptides, which are abundant in human carcinoma cells of the breast, ovary, pancreas, colon and lung (38,45). The humoral immune response to mucins lead to the survival of breast carcinoma patients during the early stages of the disease (46). In addition, Tn antigens are common between cancer and hydatid cyst and they may affect cancer growth and antitumor activity (47).

In the present study, we observed a reduction in both tumor volume and carcinogenesis in breast tissue in mice injected with 300 µg/ml of HCF and 500 µg/ml of AgB. Furthermore, our histological observations suggested that these concentrations of HCF and AgB affect breast cancer by altering histological changes, confirming their antitumor potential. However, based on our cytokine assay, the anti-carcinogenic activity of 300 µg/ml of HCF was more efficient than 500 µg/ml of AgB. In the same way, in a study on mice melanoma tumor, injection of hydatid cyst antigens could inhibit tumor growth (48).

The ratio of Th1 to Th2 cells (Th1/Th2 balance) is derived from a hypothesis suggesting that mouse Th cells express different cytokine pathways (49). We revealed that intraperitoneal injection of hydatid cyst components in mice can be similar to the condition when a cyst ruptures within the host's body, in which the Th1/Th2 ratio is polarized toward the Th1 response. On the other hand, the anticancer effect of hydatid cyst is probably related to the Th1 type immune response developed during specific stages of infection (50).

Conclusion

HCF and AgB have a prophylactic impact on breast cancer. The reduced tumor size detected in AgB and HCF groups may be connected with the role of the Th1 immune response in reducing tumor cell growth. The possible antitumor effect of parasites may be due to the mutual antigenic compositions between these infectious pathogens and cancer leading to induction of innate or acquired immunity to activate anti-angiogenesis pathways. Thus, parasitic antigens can be applied for prophylactic approaches or immunotherapy of different types of malignancies such as breast cancer. However, more comprehensive and precise experiments are required to investigate the relevant anticancer mechanism.

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Competing interest

The authors declare that they have no conflict of interest.

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