



Investigating the effect of cGRP78 vaccine against different cancer cells and its role in reducing melanoma metastasis

Hamed Zare¹, Hamid Bakherad^{2,3,*}, Arman Nasr Esfahani², Hossein Aghamollaei⁴,
Seyed Latif Mousavi Gargari⁵, Mahdi Aliomrani⁶, and Walead Ebrahimizadeh⁷

¹Recombinant Proteins Department, Breast Cancer Research Center, Motamed Cancer Institute, ACECR, Tehran, Iran

²Department of Pharmaceutical Biotechnology, Faculty of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.

³Pharmaceutical Sciences Research Center, Isfahan University of Medical Sciences, Isfahan, Iran.

⁴Chemical Injuries Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran.

⁵Department of Biology, Faculty of Basic Science, Shahed University, Tehran, I.R. Iran.

⁶Department of Pharmacology and Toxicology, Isfahan Pharmaceutical Science Research Center, Faculty of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.

⁷Department of Surgery, Division of Urology, McGill University and the Research Institute of the McGill University Health Centre (RI MUHC), Montreal, Quebec, Canada.

Abstract

Background and purpose: Treatment of malignancies with chemotherapy and surgery is often associated with disease recurrence and metastasis. Immunotherapy improves cancer treatment by creating an active response against tumor antigens. Various cancer cells express a large amount of glucose-regulated protein 78 (GRP78) protein on their surface. Stimulating the immune system against this antigen can expose cancer cells to the immune system. Herein, we investigated the effectiveness of a cGRP78-based vaccine against different cancer cells.

Experimental approach: BALB/c mice were immunized with the cGRP78. The humoral immune response against different cancer cells was assessed by Cell-ELISA. The cellular immunity response was determined by splenocyte proliferation assay with different cancer antigens. The effect of vaccination on metastasis was investigated in vaccinated mice by injecting melanoma cancer cells into the tail of mice.

Findings/Results: These results indicated that the cGRP78 has acceptable antigenicity and stimulates the immune system to produce antibodies. After three injections, the amount of produced antibody was significantly different from the control group. Compared to the other three cell types, Hela and HepG2 showed the highest reaction to the serum of vaccinated mice. Cellular immunity against the B16F10 cell line had the best results compared to other cells. The metastasis results showed that after 30 days, the growth of B16F10 melanoma cancer cells was not noticeable in the lung tissue of vaccinated mice.

Conclusion and implications: Considering the resistance of vaccinated mice to metastasis, this vaccine offers a promising prospect for cancer treatment by inhibiting the spread of cancer cells.

Keywords: cGRP78 vaccine; Metastasis; Melanoma; Immunotherapy.

INTRODUCTION

The initial stages of malignancy treatment include chemotherapy, surgery, and radiotherapy. However, in most cases, there will be the possibility of disease recurrence and metastasis after these steps. One of the new research fields that creates an active response

against tumor antigens is immunotherapy against cancer (1). Immunotherapy activates the immune system against cancer surface antigens, and T cells play a special role in this mechanism (2).

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*Corresponding author: H. Bakherad
Tel: +98-3137927055, Fax: +98-3136680011
Email: bakheradh@pharm.mui.ac.ir

On the other hand, cancer cells can escape from the immune system by hiding their antigens from T cells. Therefore, one of the most important goals of immunotherapy in cancer treatment is to create tumor-specific T cells (3). One of the new trials in the prevention and treatment of malignancies that uses immunotherapy mechanisms is the use of surface antigens of cancer cells to develop cancer vaccines (4). The main goal of anti-tumor vaccines is to induce an appropriate and long-lasting immune response against tumors, which is able to stop the recurrence of the tumor. An effective vaccine that can better treat cancer cells should be able to rouse cellular and humoral immune responses against cancer cells (1,5,6). The production of heat shock protein (HSP) housekeeping proteins, which are produced in the cells of all living organisms, increases in some pathological conditions such as hypoxia or heat shock (7). The high rate of proliferation in cancer cells continuously leads to a lack of nutrients and oxygen (hypoxia) in these cells. As a result, members of the Hsp70 families increase their expression in these cells, particularly the extracellular and membrane-bound cells, and migrate there more than they normally do (7). In the following, antigen-presenting cells are activated against these antigens and play their role in cancer immunotherapy.

Glucose-regulated protein 78 (GRP78) is an active chaperone in the endoplasmic reticulum and a member of the HSP70 family. The most important function of GRP78 is in regulating intracellular transport of proteins, receptor-dependent endocytosis, and apoptosis. Another activity of GRP78 is targeting misfolded proteins for proteasomal degradation and regulating calcium homeostasis in cells (8,9). Many articles have reported high expression of GRP78 on the surface of cancer cells (10-14). In cancer cells and stressed cells, the high expression of GRP78 in the endoplasmic reticulum causes the migration of this protein to the cell surface, which rarely happens in normal cells. The amount of GRP78 on the cell surface is directly related to the growth rate, malignancy, anti-apoptotic activity, drug resistance, and metastasis of cancer cells (9,15,16).

Various cancer cells, such as breast cancer, melanoma, hepatoma, osteosarcoma, and pancreatic cancer, express a large amount of GRP78 protein on their surface (17,18). In the study by Cai *et al.*, the role of GRP78 in the chemotherapy process of breast cancer was confirmed using *in vivo* breast cancer xenografts, which makes this antigen an attractive candidate for use in immunotherapy and vaccination (10). Moreover, there is a lot of evidence showing that GRP78 is closely related to the progression and poor prognosis of lung cancer. Therefore, this protein has a significant role in lung cancer therapy and can potentially be considered a suitable candidate for lung cancer vaccination (11,19). On the other hand, several studies have revealed that GRP78 may play an important role in the development and progression of hepatocellular carcinoma. In melanoma cancer cells, increased surface expression of GRP78 is associated with drug resistance and malignant progression (14). It has been found that overexpression of cell surface GRP78 boosts the proliferation, growth, and migration of cancer cells, and the suppression of this receptor has reduced metastasis to the lung and increased the survival of the tested mice (11,19,20). These findings indicate cell surface GRP78 as an encouraging cancer cell-specific biomarker and a beneficial target for the treatment and imaging of tumor cells (17,18).

Many studies show that antibodies that bind to the N-terminal domain of GRP78 increase the growth and metastasis of tumor cells, but antibodies against the C-terminal domain of this protein inhibit the growth and metastasis of cancer cells (21,22). This shows the importance of choosing cGRP78 as a suitable target for removing cancer cells. Producing antibodies against this protein or using it as a vaccine against cancer cells can open new horizons in cancer treatment.

In previous studies, our investigations using bioinformatics and laboratory data showed that the production of the C-terminal domain of the GRP78 protein maintained its original structure (21). This truncated recombinant protein can be a suitable candidate as an anticancer vaccine because it causes the immune system to respond against the C-terminal protein GRP78, which

scientists have shown will lead to the destruction of cancer cells. Unlike most cancer-specific antigen vaccines, a cGRP78-based vaccine can act against many cancer cells, and depending on the level of surface expression of GRP78 on cancer cells, it can eliminate or reduce the growth and metastasis of cancer cells.

This study aimed to investigate the reaction of a cGRP78-based vaccine on various cancer cells, such as hepatoma, lung, breast, melanoma, and cervical cancer, and to evaluate its effect on preventing the metastasis of melanoma cancer cells (B16F10) in a mouse model.

MATERIALS AND METHODS

Cell lines and mice

Cancer cell lines including 4T1, B16F10, HepG2, A549, Hela, and normal cell line including HUVEC were purchased from the cell bank of the Pasteur Institute of Iran and cultured in DMEM and RPMI culture mediums enriched with 10% fetal bovine serum (FBS; DNAbiotech Co., Iran) in a humidified incubator under 5% CO₂ at 37 °C depending on the cell types (23). The cells were passaged at the appropriate time and frozen for later use.

The mice used in this study were 4-week-old BALB/c males purchased from Royan Laboratory Animal Center of Isfahan, kept in standard environmental and nutritional conditions. All the steps of working with mice have been done according to the Animal Welfare Guidelines, and the proposal of this research was approved by the Institutional Ethical Committee and Research Advisory Committee of Isfahan University of Medical Sciences in Iran (Ethical No. 1398.849).

Preparation of cGRP78

Cloning of cGRP78 recombinant protein has been performed in our previous study (21). The gene for this recombinant protein (690 bp) was cloned in the pET22b vector. In the following, its purification was carried out with the help of the histidine tag and by the nickel-nitrilotriacetic acid (Ni-NTA) column. The expression of this recombinant protein was done at 30 °C for 16 h, and its purification

was performed by a native purification protocol (Invitrogen). Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to evaluate the purity of the recombinant protein, and a western blot with an anti-GRP78 antibody (Abcam Co., UK) was used to confirm it. SDS-PAGE and western blot were performed according to the procedure mentioned in the previous article (24).

Mouse immunization with cGRP78

To investigate the response of the humoral and cellular immune systems of mice vaccinated with the recombinant protein cGRP78 against different cancer cells, 4-week-old BALB/c mice (n = 16) were randomly divided into two groups. The mice of the test group were injected with 0.1 mL of 500 µg/mL cGRP78 recombinant protein (50 µg/mice) in three boosters with a ten-day interval, complete and incomplete Freund's adjuvant as a vaccine. Also, phosphate-buffered saline (PBS; DNAbiotech Co., Iran) was injected in three boosters with complete and incomplete Freund's adjuvant in the control group. The injection was performed peritoneally in the left and right flanks of the mice. Before each injection, blood was taken from two mice from the test and control groups *via* a capillary tube of the left eye to track the antibodies produced in the mice's bodies against cGRP78 using the enzyme-linked immunosorbent assay (ELISA) technique. After finishing the vaccination of mice, 4 mice from each group were used to collect blood and isolate serum to perform the humoral immunity test against cancer cells. Moreover, 4 mice were sacrificed and their spleens were used to perform the cellular immune response test against cancer cells.

Evaluation of the immunized mice serum response to different cancer cells

Antibodies against cGRP78 in the sera of immunized mice were assayed by ELISA. Firstly, the cGRP78 protein was prepared with a final concentration of 50 µg/mL in the coating buffer (carbonate bicarbonate buffer, pH 9.4). Then, 100 µL of it was added to each well in 96-well plates (5 µg of protein in each well). The plate was incubated at 37 °C for 16 h. After washing with PBST (PBS buffer with 0.1%

tween 20), 100 μ L of blocking buffer (5% skim milk; Merck, Germany) in PBS buffer) was poured into each well to block the wells. Serial dilutions were prepared from the sera of immunized and control mice (1:100, 1:200, 1:500, and 1:1000) and exposed to cGRP78 for 1 h with triplicate repetitions. After washing, horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Sigma-Aldrich, USA) with a dilution of 1:50,000 was added in the wells, and 3, 3', 5, 5' tetramethylbenzidine (TMB; DNAbiotech Co., Iran) was used to track the bound antibodies. At the end of the reaction, after stopping the reaction with H₂SO₄ (0.16 M; Merck, Germany), absorbance was measured at 450 nm with the ELISA Reader (Bio-Rad, model 680, California, USA).

After confirming the production of specific antibodies against cGRP78 recombinant protein in immunized mice, the serum of these mice was collected, and the reaction of the serum antibodies with different cancer cells was evaluated by the cell-ELISA method. Each 96-well plate was cultured with 30,000 different cancer cells, including 4T1, HepG2, A549, Hela, and HUVEC cells as a negative control, and B16F10 cells as a positive control, for 24 h. B16F10 cells have shown high surface expression of GRP78 in different references, so it is suitable as a positive control (25). Paraformaldehyde (Merck, Germany) with a concentration of 4% was added to each well and incubated for 15 min at room temperature. After collecting the medium and washing wells, a 5% skim milk (Merck, Germany) solution was used for blocking overnight at 4 °C. After washing three times, serum obtained from immunized and control mice was used with two different dilutions (1/500 and 1/1000) with three repetitions on different cancer cells, and the control cells were incubated for 1 h at 37 °C in a shaker incubator. After washing three times, 100 μ L of goat anti-mouse HRP-conjugated antibody was added to the wells and incubated for another hour in a shaker incubator at 37 °C. TMB was used to evaluate the number of antibodies attached to the surface of the cells, and after stopping the reaction, the results were estimated by an ELISA reader at 450 nm.

Splenocyte proliferation response with different cancer cell antigen

The growth-stimulating potency of cancer cell antigens on cGRP78-immunized mouse spleen lymphocytes was evaluated by the MTT test. The isolated splenocytes of the vaccinated and control groups were cultured at a density of 1×10^5 cells per well in a 96-well plate with 20 μ g/mL of 4T1, HepG2, A549, Hela, HUVEC (negative control), and B16F10 (positive control) cells antigens cultured in complete medium (26). Cancer cell antigens were prepared by freeze-thawing method (27). After 72 h, MTT solution (DNAbiotech, Iran) was added to the wells and incubated for 4 h. Then, the medium on the cells was removed, and dimethyl sulfoxide (DMSO; DNAbiotech, Iran) was used to dissolve the formazan crystals. The amount of color produced was evaluated at a wavelength of 570 nm with an ELISA reader. All experiments were performed in triplicates.

In vivo metastatic tumor treatment

According to the results of humoral and cellular immunity tests on different cancer cells, the melanoma cancer cell B16F10 was selected for an *in-vivo* metastasis test. Four-week-old BALB/c mice were randomly divided into test and control groups (8 mice in each group). Then, 5×10^4 B16F10 cells were injected into all mice through the tail vein. Immediately after the injection of cancer cells, the mice in the test group were vaccinated with the cGRP78 protein in three boosters with an interval of ten days. The control group was injected with PBS instead of cGRP78 protein. The first injection of vaccine and PBS (in the control group) was done with complete Freund's adjuvant, and the subsequent injections were done with incomplete Freund's adjuvant. After one month, all mice were sacrificed according to the Animal Welfare Guidelines (28), and their spleen, lung, and liver were examined for black cancerous nodules of B16F10 (29).

Statistical analysis

All experiments were performed three times. Data obtained from different experiments were

analyzed using a one-way ANOVA followed by Tukey post-test. Also, a significant difference between the two groups was found by Student's t-tests. All statistical analysis was performed using SPSS software (version 26.0; SPSS Inc.) and GraphPad Prism 9. P -values ≤ 0.05 were considered statistically significant.

RESULTS

Expression and purification of the cGRP78 protein

The expression of the cGRP78 protein was investigated under different conditions, and the optimal expression was obtained 16 h after induction with 0.5 mM IPTG at 30 °C. Purification of the recombinant protein was performed under native conditions using a Ni-NTA column. Native purification condition allows the resulting protein to retain its structure and do not require refolding. The purified 28-kDa band in SDS-PAGE indicates that the cGRP78 protein was obtained with appropriate purity. The western blot technique was used to confirm the obtained protein (Fig. 1A and B).

Humoral immunity reaction with different cancer cells

Specific antibodies against recombinant cGRP78 were induced in BALB/c mice after vaccination. As shown in Fig. 2, after three injections, the amount of antibody produced (mean of OD ~ 1.13) is significantly different from the control group (comparison of means with a t-test, P -value 0.001).

In the next step, the reaction of the immunized mouse serum and the control serum after the third injection was analyzed on different cancer cells coated on the ELISA plate. Its results are shown in Fig. 3. In this test, two human cancer cells (A549 and Hela) and three mouse cancer cells (4T1, B16F10, and HepG2) were used. Examining the obtained results showed that, despite the use of human cells and the possibility of a non-specific mouse antibody reaction with these cells, the serum antibodies acted specifically and identified the cGRP78 antigen. The results of this test indicated that Hela (mean of OD ~ 0.72) and HepG2 (mean of OD ~ 0.68) cells had the highest reaction with antibodies obtained from vaccinated mice (Fig. 3). Comparison between three groups with One-way ANOVA, P -value 0.01.

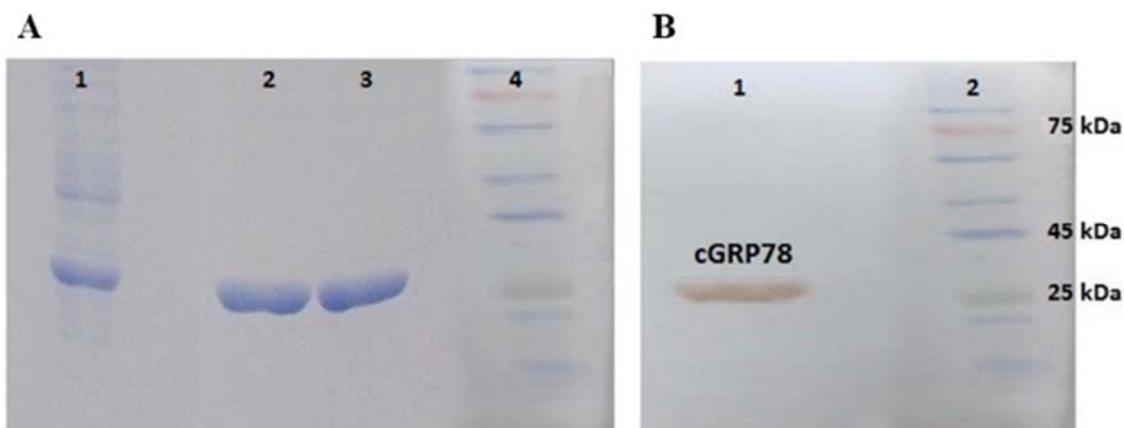


Fig. 1. The SDS-PAGE and western blotting of cGRP78 protein. (A) Expression and purification of cGRP78 recombinant protein. Line 1: soluble fraction from protein expression in *E. coli* before purification; lines 2 and 3: recombinant protein cGRP78 after purification by Ni-NTA; line 4: PageRuler™ plus Prestained Protein Ladder. (B) Western blotting of cGRP78. Line 1: purified protein; line 2: Prestained Protein Ladder. SDS-PAGE, Sodium dodecyl-sulfate polyacrylamide gel electrophoresis; GRP, glucose-regulated protein 78; Ni-NTA, nickel-nitrilotriacetic acid.

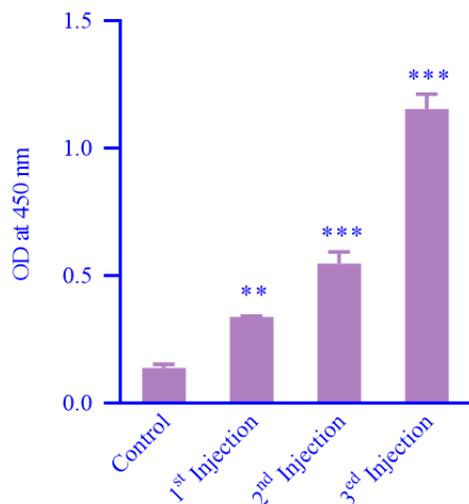


Fig. 2. Anti-cGRP78 IgG titer after mouse immunization. OD represents the amount of produced antibody. ** $P < 0.01$ and *** $P < 0.001$ indicate significant differences in comparison with the control group. GRP, glucose-regulated protein 78; OD, optical density.

Cellular immune response and splenocyte proliferation induced by cGRP78

To evaluate the induction of cell-mediated immunity after injection of the cGRP78 vaccine, the proliferation ability of splenocytes exposed to different cancer cell antigens was investigated. Splenocytes obtained from vaccinated mice and the control group were isolated and cultured. The proliferation of splenocytes in the presence and absence of five cancer cell antigens was measured after 72 h using the MTT assay. Also, as a control group, antigens of a normal cell (HUVEC) were used

instead of cancer cell antigens. The results shown in Fig. 4 confirm that the B16F10 antigens more than other cancer cell antigens increased the proliferation of splenocytes (mean of OD ~ 0.9) in vaccinated mice compared to its respective control group. Also, the comparison of the cellular immune status of vaccinated mice in the “vaccinated + antigen group” (vaccinated mouse splenocyte along with cancer antigens) and the “vaccinated - antigen group” (vaccinated mouse splenocyte in the absence of cancer antigens) showed that the proliferation of splenocytes of immunized mice in the presence of antigen was significantly higher than when there was no antigen in the environment.

In vivo tumor treatment experiments

Considering that in the cellular immunity test, B16F10 cells stimulated cellular immunity more than other cancer cell lines, to evaluate the capability of the cGRP78 vaccine in the prevention of B16F10 metastasis, cancer cells were injected into the blood of cGRP78-vaccinated and control mice. The lung, liver, and spleen tissues of mice in both groups were examined for cancer nodules. The results showed that after 30 days, the growth of cancer cells was not noticeable in the lung, liver, and spleen tissues of cGRP78-vaccinated mice, but the mice in the control group contained black cancerous nodules resulting from the growth of B16F10 cells. The results of comparing the lungs of vaccinated mice and the control group are shown in Fig. 5.

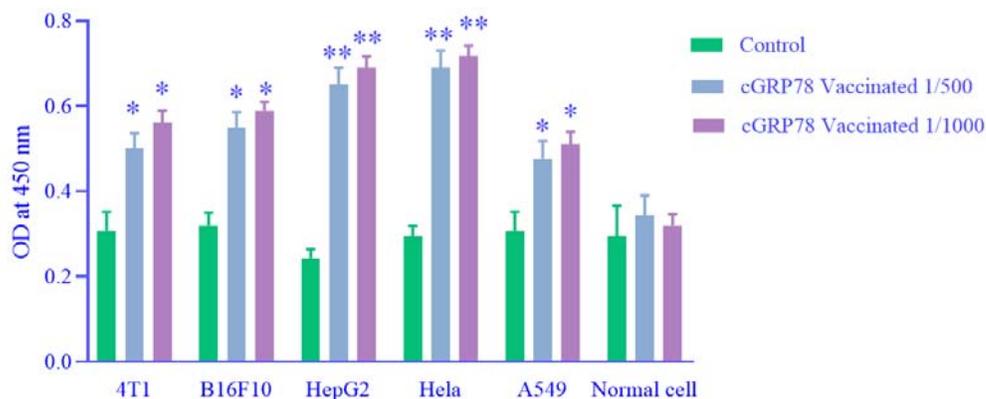


Fig. 3. Humoral immune status of immune mice against different types of cancer cells. Two human cancer cells (A549 and Hela) and three mouse cancer cells (4T1, B16F10, and HepG2) were used to examine the humoral immunity in vaccinated mice. Hela and HepG2 cells reacted the most with the serum of vaccinated mice. * $P < 0.05$ and ** $P < 0.01$ indicate significant differences compared to the respective control group. OD, Optical density.

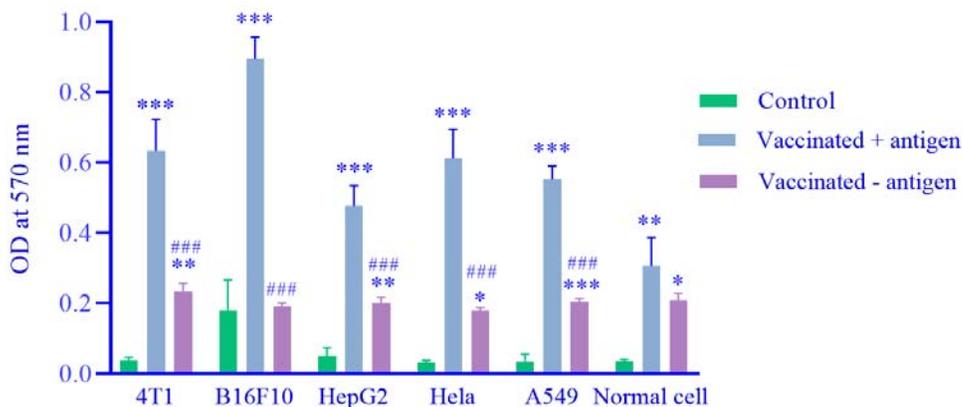


Fig. 4. Cellular immune status of vaccinated mice against different types of cancer cells in the “vaccinated + antigen group” (vaccinated mouse splenocyte along with cancer antigens) and “vaccinated - antigen group” (vaccinated mouse splenocyte in the absence of cancer antigens). The B16F10 cells’ antigens more than other cancer cell antigens increased the proliferation of splenocytes in vaccinated mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ indicates significant differences compared to the respective control and ### $P < 0.001$ indicates the differences between vaccinated groups. OD, Optical density.

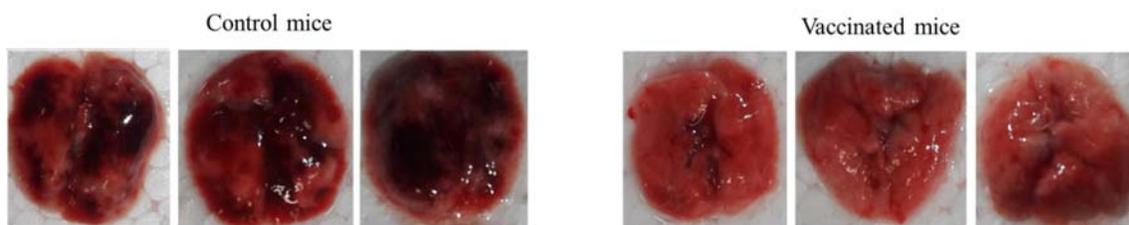


Fig. 5. Investigating lung metastasis of melanoma cells in vaccinated mice. The black nodules in the lungs of control group mice indicate the metastasis of cancer cells in the lungs.

DISCUSSION

GRP78, as a member of the HSP70 family, prevents the aggregation of unfolded proteins by binding to hydrophobic residues in the ER. Interestingly, due to the GRP78 overexpression in cancer tissues and its emergence on the surface of cancer cells, this antigen is a potential target for cancer immunotherapy (30).

In a wide range of cancers such as lung (10,20), hepatocellular (12), breast (11,19,31), cervical (13), bladder (22), melanoma (14), gastrointestinal tract (2,32), and ovary (21), overexpression of GRP78 induces resistance to numerous drug agents. Therefore, it is possible to increase the sensitivity of tumors to drug treatment through immunotherapy and by inhibiting GRP78. As a result, this method can limit the growth rate of tumors.

The production of antibodies against different epitopes of GRP78 has been done by

different researchers, and the results have been reported in the literature. In a study conducted by Misra *et al.* antibodies were raised against the GRP78 carboxyl domain-induced apoptosis in prostate and melanoma cells (33). In another study, performed on colon and breast cancer cells, the use of the high-affinity monoclonal antibody Mab-159 (KD = 1.7 nmol/L) against surface GRP78 inhibited tumor cell proliferation (34).

In our previous investigation, the recombinant C-terminal domain of GRP78 protein was evaluated by bioinformatics tools and produced in *E. coli*. Experimental evaluations of this antigen by circular dichroism and ELISA methods revealed that the two- and three-dimensional structures of both recombinant and native proteins are very similar to each other. For this reason, this truncated recombinant protein can be an interesting candidate for vaccination against cancer (21).

The humoral immunity capacity of immunized mice against various cancer antigens was tested using cell ELISA after vaccination with the cGRP78 protein. The results indicate that the cGRP78 protein has acceptable antigenicity and stimulates the immune system to produce antibodies.

Vaccination with this recombinant antigen stimulates humoral immunity against cancer cell antigens, and the serum antibodies identified A549, HeLa, 4T1, B16F10, and HepG2 cell lines. It should be noted that HeLa and HepG2 cells showed the highest reaction with the serum of vaccinated mice, which may be due to the high expression of GRP78 at the level of these two cell lines (19,35).

The stimulatory effect of cancer antigens on the proliferation ability of splenocytes from immunized mice was investigated after vaccination by cGRP78. This antigen significantly increased the proliferative excitability in splenocytes of the vaccinated mice compared to non-vaccinated mice. This shows the stimulating effect of the cGRP78 vaccine on the proliferation of immune cells (36). According to the results, it can be concluded that the cellular immune response reacts more to the B16F10 cancer cell line and the probability of removing this cell is higher by the cellular immune system.

Considering that the role of cellular immunity in cancer immunotherapy is more important than humoral immunity, and on the other hand, the obtained results show high cellular immunity against melanoma cells, B16F10 (a melanoma cell line) was used for *in vivo* testing and further investigation.

In a study by Zhang *et al.* four-weak B16F10 neoantigens were used as vaccine targets. These antigens fused to the diphtheria toxin, and this recombinant vaccine elicited anti-tumor CD8+ T cell responses. "DTT-neo Ag" vaccine inhibited tumor growth at a rate of 88% and 100% in the preventive and therapeutic models, respectively (37). In another study, an mRNA vaccine developed was able to significantly and specifically protect mice against B16F10 melanoma tumor progression. This mRNA vaccine elicited a cellular immune response characterized by the production of interferon-gamma and the induction of cytotoxic

T lymphocytes (38). In our study, the proliferation of splenocytes in the presence of cancer cell antigens showed that the recombinant GRP78 vaccine is also able to stimulate cellular immunity. Moreover, the results of the metastasis of B16-F10 melanoma cells in our investigation showed that vaccinated mice with cGRP78 were more resistant to melanoma metastasis, and the immune systems of these animal models were more effective in inhibiting the spread of cancer cells. Among the limitations of this project, we can mention the lack of appropriate tumorigenesis in mice and the ethical problems caused by working on laboratory animals. By using tissue engineering and designing *in vitro* tests suitable for tumorigenesis, these problems can be solved to some extent.

CONCLUSION

The results of our studies on vaccination by cGRP78 can open a new approach to the clinical management of melanoma cancer patients. This *in vivo* investigation clarified that cGRP78 could be an interesting target for additional research in melanoma cancer therapy. Moreover, considering the acceptable results of this vaccine in inhibiting melanoma metastasis in a mouse model, which is caused by the effective stimulation of cellular immunity, this vaccine can be used for other cancer cell lines that have good results in stimulating cellular immunity (such as 4T1 and HeLa). However, it is necessary to perform more detailed tests *in vivo* on other cell lines used in this research.

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Conflict of interest statement

All authors declared no conflict of interest in this study.

Authors' contributions

H. Bakherad carried out most of the experiments including making the constructs,

contributed to the design and the supervision of the study, analyzed the data and prepared the figures; H. Zare wrote the manuscript and conducted the statistical analysis; A. Nasr Esfahani carried out vaccine preparation, administration, and results; H. Aghamollaei supervised the pathologic experiments; S.L. Mousavi Gargari provided scientific comments and suggestions; M. Aliomrani provided laboratory animals. W. Ebrahimzadeh modified the text and corrected the manuscript. The finalized article has been approved by all authors.

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