GENERATION OF ANTI-IDIOTYPIC RESPONSES AGAINST AN85KDA BREAST TUMOR ASSOCIATED ANTIGEN: AN IMMUNOTHERAPEUTIC APPROACH

Ishita Mandal[1] and Rathindranath Baral[2]

[1]Molecular Biology Department, NetajiSubhas Bose Cancer Hospital, Kolkata, India
[2]Immunoregulation and Immuno diagnostics Department, Kolkata, India

ABSTRACT

An 85KDa Breast tumor Associated Antigen (BTAA) was recognized and distinguished partly, from human malignant breast tissue. The use of neem leaf glycoprotein extract (NLGP) in increasing the antigenicity of BTAA has already been tested and NLGP was observed to enhance the antigenicity of BTAA considerably. In this work, it was shown that the anti-idiotypic antibody could also be generated against BTAA. The raising of antigenicity of BTAA by NLGP, as well as the generation of anti-idiotypic responses, has implications in the immunotherapy of breast cancer.

KEYWORD

BTAA, NLGP, Idiotypic, Antibodies

1. INTRODUCTION

Active-specific immunotherapy is becoming out to be one of the important methods of cancer immunotherapy. Identification of tumor-associated antigens is important for this mode of immunotherapy. Many tumor-associated antigens (TAA) have been identified to date, which could be theoretically considered good for active specific immunotherapy [1, 2]. However, antibodies are not developed against some TAAs. This is supposed to be due to insufficient immunogenicity of the antigens [3, 4]. The immunogenicity of an antigen is known to be influenced by foreignness, molecular size, chemicalcomposition, and degradability of the antigen. As most of the TAAs are developed from humans they are recognized as self. This problem could be solved by two approaches, one is the use of adjuvants [5] and another is through the anti-idiotypic pathway [6].

The idiotypic network theory was established by N. K. Jerne, for which he was awarded the Nobel Prize in 1984 with G.J. Kohler and C. Milstein. According to this theory, the structural determinants on the variable regions of a given antibody (Ab1) can also serve as determinants that are recognized by a second antibody (Ab2). This structural determinant is called idiotope [7]. Sometimes, this idiotope may be the same region that recognizes the antigenic determinant, and in some cases, it could be another region within the variable region of the antibody. There could be multiple idiotopes on an Ab1 that are recognized by an Ab2. The Ab2 is called an anti-idiotypic antibody. Ab2 can be grouped into three types (Ab2 alpha, Ab2 beta, and Ab2 gamma) based on the part of the variable region of Ab1 they recognize. Ab2 alpha recognizes idiotopes that are outside the antigen-binding site. Ab2 beta recognizes the binding site of Ab1 and resembles the original epitope recognized by Ab1. If the target idiotype is close to the antigen binding site of the antibody it may be a hindrance to antigen binding. This type of anti-idiotypic

antibody is called Ab2 gamma. As, Ab2 beta resembles the antigen, it could be used as an alternate TAA.

This idiotypic network revealed a new field of research for the control of humoral immune response. According to the network theory thesurrogate antigen will be foreign, and thus the problem of tolerance will be overcome. Soon, thereafter many researchers worked on developing anti-idiotypic antibodies. When an immune response was found to be developed in animal models due to anti-idiotypic antibodies, clinical trials were also carried out [8]. Favorable results were found in patients whose immune responses arose on administration of the vaccine [9, 10, 11].Some more recent research has been carried on to develop anti-idiotypic antibodies against cancer antigens [12, 13, 14, 15]. To date, no anti-idiotypic antibody has come into clinical practice, but the concept of surrogate antigen has led researchers towards investigations that could help in developing therapeutic regimens in the field of cancer vaccinations [16] and this work is still being carried out.

This problem of foreignness is expected to be true in the case of 85KDa Breast Tumor Associated Antigen (BTAA), also as it was purified and partially characterized earlier in the Immunoregulation and Immunodiagnostics laboratory, CNCI [17], from human malignant breast tissue. This issue could be overpowered by the use of adjuvants or by the use of anti-idiotypic antibodies. Earlier, we have reported using NLGP as an adjuvant for BTAA [18]. In this set of experiments, it was tested whether the anti- idiotypic response could also be generated against BTAA.

2. MATERIALS AND METHODS

2.1. Neem Leaf Glycoprotein

Neem leaves of the same size and age were obtained from a source of proper quality. Neem leaves of the same age were taken because the rate of photosynthesis reduces with an increase in the age of the leaves. This results in a difference in the carbohydrate levels of the leaves. Neem leaves should be taken from the same source, otherwise the micronutrients may vary. After collection, they were shed dried, and crushed. This neem leaf powder was stored at 4 degrees till further use. Before use, 0.5 mg of this powder was soaked in 1 ml PBS overnight with shaking. This extract was then centrifuged and the supernatant was collected and filtered. The endotoxin content of the filtrate was tested by the Limulus Amebocyte Lysate test. The endotoxin content was found to be negligible. This preparation was used for further experiments.

2.2. Preparation of Malignant Breast Tumor Tissue Antigen

Breast tumor-associated antigen (BTAA) was purified by the method described earlier [17]. In brief, human malignant breast tumors were collected in 0.15M cold phosphate buffer saline (PBS), minced, and homogenized. The homogenate was centrifuged successively at 6000g for 30 mins. and 15000g for 1 hour, both at 0^{o}C. The supernatant was purified by DEAE-cellulose ion exchange chromatography and the resultant was further purified by Reverse phase-high performance liquid chromatography using Protein- Pac 300 SW column (Waters).The protein content of this antigen preparation was estimated by the Bradford reagent. Purification of the antigen in each step was guided by SDS polyacrylamide gel electrophoresis.

2.3. Animals and Immunization

Dry pellets and water were given ad libitum. Female Swiss mice were obtained from the Animal Care and Maintenance Department of Chittaranjan National Cancer Institute. They were maintained under standard laboratory conditions and according to the guidelines set up by the Institutional Animal Care and Ethics Committee.

Swiss mice were immunized with BTAA with and without NLGP four times at an interval of one week. Mice immunized with PBS were considered as controls. After one week of every immunization, the serum of all the mice were saved. Antibody levels in the serum were tested and compared in the three groups of mice. IgG subclasses were studied and the efficacy of this antibody was tested earlier [18]. IgG was purified from the serum of mice immunized with BTAA+NLGP, which was found to be effective in raising immunity. Purification was done by Protein-A Sepharose 4B column chromatography.

2.4. Purification of IgG by Protein-A Sepharose 4B Column Chromatography and Immunization

Protein-A Sepharose 4B was soaked in borate-buffer saline (BBS) overnight at 4ºC. It was packed and properly equilibrated with BBS. Then, the diluted serum was loaded and flow-through was collected, and then, the bound IgG was eluted by 0.1M citrate buffer and the eluted fractions were dialyzed against PBS. IgG (Ab1 and Ab2) was purified from BTAA+NLGP and Ab1 treated mice sera respectively by Protein- A Sepharose column chromatography. Swiss mice and rats were immunized with purified IgG (Ab1) in the presence and absence of NLGP for four weeks at an interval of one week. Blood was collected one week after each immunization.

2.5. Detection of Anti-Idiotypic and Anti-Anti-Idiotypic (Ab3) by ELISA

The purified IgG was coated on plates and ELISA was performed to test whether anti-idiotypic antibody (Ab2) was generated in the immunized animals. In brief, the microtiter plates were blocked with 5% BSA. Serially diluted samples of control and immunized animal sera were added to the wells in triplicate and kept for 2 hours. The plates were washed with PBS containing 0.05%Tween-20 and goat anti-mouse IgG labeled with peroxidase (Sigma, St. Louis) was added (1:1000). Colour was developed by using Tetramethylbenzidine (TMB) and measured at 450 nm using a microtiter plate reader (Tecan Spectra, Grodig, Austria). Ab2 was then used for immunizing the animals in the presence and in the absence of NLP for the production of Ab3. Blood was collected from these animals and the serum was isolated, stored, and tested for Ab3 production.

2.6. Inhibition Assay

2.6.1. Inhibition Assay 1 (Inhibition Of Binding Between Ag and Ab1)

Purified BTAA (5µg/ml) was coated in plates and kept overnight. The plate was blocked and washed. 25µl of Ab1 (1:50 dil) was co-incubated with 25µl of the Ab2 sera (non-immune and immune) in different dilutions (1:200,1:400,1:800, 1:1600). Non-immune sera and Ab1 sera were used as negative and positive controls. The plate was incubated for 2hours at room temperature and then washed with PBS-Tween20. Secondary Ab was added (anti-mouse IgG labeled with alkaline phosphatase) at 1:1500 and kept for 1hour. The plate was again washed with PBS-T and the substrate solution was added (p-nitrophenylphosphate dissolved in diethanolamine buffer). The developed color was determined by measuring optical densities at

405 n.m

2.6.2. Inhibition Assay 2 (Inhibition of Binding Between Ab1 And Ab2)

Purified Ab1 (5µg/ml) was coated in plates and kept overnight. The plate was blocked and washed. 25µl of Ab2 (1:50 dil) was co-incubated with 25µl of the Ab3 sera (non-immune and immune) in different dilutions (1:200,1:400,1:800, 1:1600). Non-immune sera and Ab2 sera were used as negative and positive controls. The plate was incubated for 2hours at room temperature and then washed with PBS-Tween20. Second Ab was added (anti-mouse IgG labeled with alkaline phosphatase) at 1:1500 and kept for 1hour. The plate was again washed with PBS-T and the substrate solution was added (p-nitrophenylphosphate dissolved in diethanolamine buffer). When color developed, optical densities were determined at 405nm. The developed color was determined by measuring optical densities at 405 n.m.

3. RESULTS

3.1. NLGP Enhances the Generation of Ab2 (Anti-Anti-BTAA IgG)

3.1.1. ELISA

Three groups of Swiss mice were injected with PBS, Ab1 (anti-BTAA IgG-25µg), and Ab1 + NLGP respectively, four times at an interval of one week. Sera were tested for the development of Ab2 by ELISA. It was observed (Fig. 1) that the quantity of Ab2 was significantly higher in the group of mice immunized with Ab1 + NLGP than the Ab2 developed in the group immunized with Ab1 only.

3.1.2. Inhibition Assay for Anti-Idiotypic Antibody

To determine whether NLGP-generated Ab2 is site-specific anti-idiotypic antibody, the binding of Ab1 tothe BTAA was measured for inhibition in the presence of Ab2 sera. It is apparent from Fig. 2 and Fig. 3 that the binding of Ab1 with BTAA was inhibited by Ab2 sera, generated by Ab1+NLP. The use of serially diluted Ab2 sera revealed that % of inhibitions decreased with dilutions when Ab1-generated Ab2sera were used. On the other hand, % of inhibitions remained steady with dilutions, when Ab1+NLP- generated Ab2 sera were used.

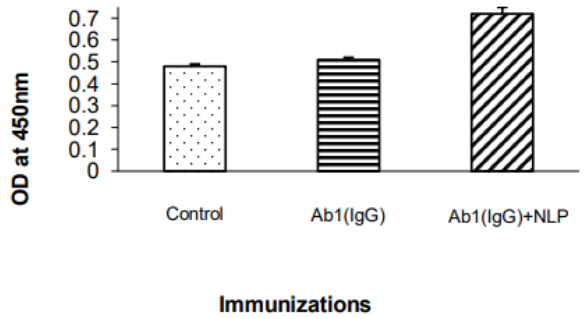

Figure1. Generation of Ab2 (anti-anti-BTAA antibody)

Fig. 1. Generation of Ab2 (anti-anti-BTAA antibody). Three groups of Swiss mice were injected with PBS, Ab1 (anti-BTAA IgG-25µg), and Ab1 + NLP respectively, four times at an interval of one week. Sera were tested for the development of Ab2 by ELISA.

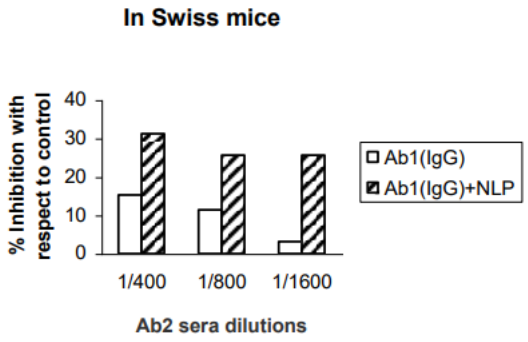

Figure 2. Inhibition of BTAA-Ab1 binding by Ab2 in Swiss mice.

Figure 2. Inhibition of BTAA-Ab1 binding by Ab2 in Swiss mice. The binding of Ab1 to the BTAA was measured for inhibition in the presence of different dilutions of Ab2 sera (anti-anti-BTAA antibody), generated in mice

Figure 3. Inhibition of BTAA-Ab1 binding by Ab2 in Sprague Dawley rats.

Figure 3. Inhibition of BTAA-Ab1 binding by Ab2 in Sprague Dawley rats. The binding of Ab1 to the BTAA was measured for inhibition in the presence of different dilutions of Ab2 sera (anti-anti-BTAA antibody), generated in rats

3.2. NLGP Enhances the Generation of Ab3

The generation of Ab3, due to immunization with Ab2, was tested by the following experiments-

3.2.1. ELISA

The generated Ab2 was purified by IgG Sepharose column chromatography as mentioned earlier, and thenwas used for the immunization of mice. The non-immune and immune sera were reacted with BTAA, which was coated in plates before, by ELISA. It was observed that Ab3 was generated when mice were immunized with IgG (Ab2) and this was slightly higher in mice immunized with both IgG (Ab2) and NLP(Fig. 4).

3.2.2. Flow Cytometry

It was observed that Ab3 could recognize the nominal antigen BTAA expressed on MCF7 cells because a considerably higher number of cells were observed to be stained in the case of mice immunized with both IgG and this was even higher in the case of mice immunized with both IgG (Ab2) and NLP (Fig. 5).

3.2.3. Immunocytochemistry

It was observed (Fig. 6) that Ab3 could recognize the BTAA expressed on MCF7 cells because much higher reaction in the case of mice immunized with IgG (Ab2) than that of control and the reaction was of the same extent as in the case of mice immunized with both BTAA and NLP. Immune reaction was mainly on the surface of MCF7 cells.

3.2.4. Inhibition Assay

Purified Ab1, was coated in plates and Ab3 was added in different dilutions with and without Ab2. Both non-immune and immune sera (mice immunized with IgG [Ab2] and both IgG [Ab2]+NLGP) were used for ELISA. It was observed from Fig. 7 `that Ab3 was able to inhibit the binding between Ab1 and Ab2.

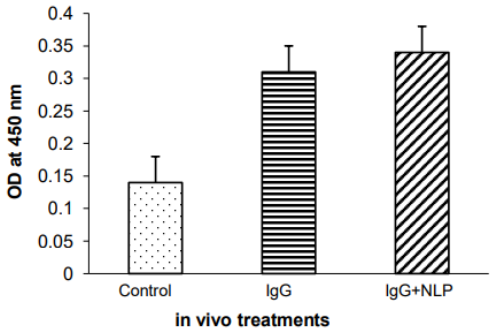

Figure. 4. Generation of Ab3 (anti-anti-idiotypic antibody) tested by ELISA

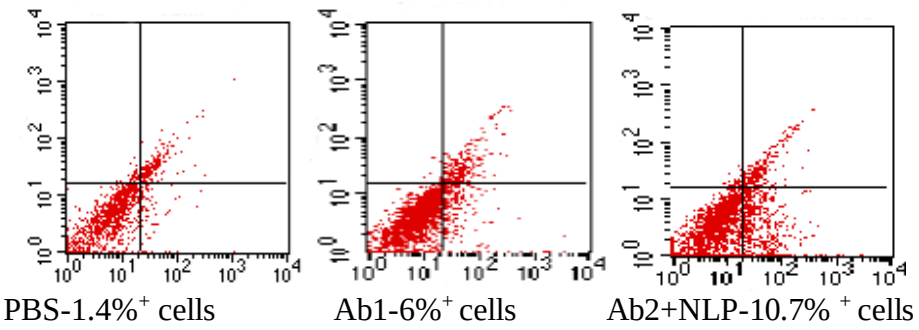

Figure. 5. Generation of Ab3 (anti-anti-idiotypic antibody) tested by flow cytometry

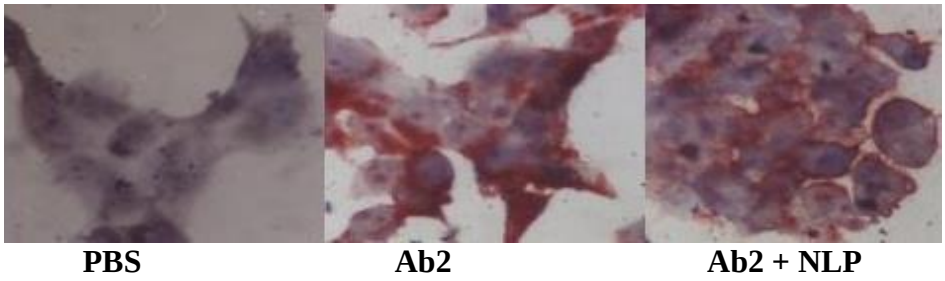

Figure. 6. Generation of Ab3 (anti-anti-idiotypic antibody) tested by Immunocytochemistry

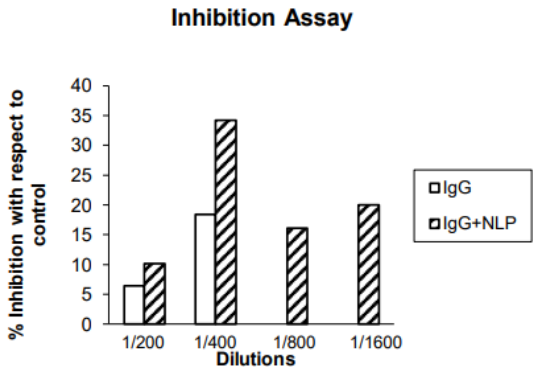

Figure. 7. Inhibition of BTAA-Ab1 binding byAb3 (anti-anti-idiotypic antibody)

Figures. (4-7). Generation of Ab3 (anti-anti-idiotypic antibody). Ab2 was purified by ProteinA- Sepharose column chromatography and used for the immunization of mice either alone or in combination with NLP.

4. Ab3 was tested by its reaction with BTAA coated on plates by ELISA.
5. Ab3 was tested by its reaction with BTAA$^+$ MCF7 cells by flow cytometry. a. PBS, b. Ab2, c.Ab2+NLP.
6. Ab3 was tested by its reaction with BTAA$^+$ MCF7 cells by immunocytochemistry. a. PBS, b.Ab2, c. Ab2+NLP.
7. The binding of Ab1 to BTAA was measured for inhibition in the presence of Ab3 sera.

4. DISCUSSION

Chemotherapy and radiotherapy are still the most widely used modes of cancer treatment. It is well known that they have some serious side effects. So, scientists throughout the world have attempted to develop many forms of targeted therapy. Developing a cancer vaccine based on a tumor associated antigen is one of the important forms of targeted therapy. In this set of experiments, we have attempted to investigate whether it is possible to generate the anti-idiotypic response with BTAA which is otherwise weakly immunogenic.

As tested earlier, immunization of mice with BTAA+NLGP resulted in a good antibody titer [18]. This antibody was purified from the immune sera and termed Ab1, which consists of idiotopes and was used to generate anti-idiotypic antibodies (Ab2). Ab1 was used either alone or in combination with NLGP, for Ab2 generation. Results (Fig. 1) showed that Ab2 response was better when Ab1 was combined with NLGP, as evidenced by the monitoring of antibody response and inhibition of binding between BTAA and Ab1. Fig. 2 shows the inhibition in BTAA-Ab1 binding by Ab2 sera in Swiss mice and Fig. 3 shows the inhibition in BTAA-Ab1 binding by Ab2 sera in Sprague-Dawley rats. The presence of inhibition in BTAA-Ab1 binding by Ab2 sera proved the generation of Ab2. As, this Ab2 could inhibit the binding between the original antigen BTAA and Ab1, it indicates that its structure is close to the original antigen and so it is Ab2β. According to the idiotypic network hypothesis of Lindenmann and Jerne [7], Ab2β is the mirror image of the nominal antigen (BTAA), and thus, used as a surrogate in the immunization protocol. BTAA is a self antigen, but, the generated anti-idiotypic antibody (Ab2β) is foreign to the human system.

We have also tested whether Ab2β is effective in generating the anti-anti-idiotypic antibody (Ab3) response. Successful generation of the Ab3 was confirmed by its binding to BTAA either isolated or on MCF7 cells. Fig 4 suggests the generation of Ab3 by its binding to isolated BTAA. Fig. 5 and Fig. 6 suggest the generation of Ab3 by its binding to BTAA on MCF7 cells. The Breast Tumor Associated Antigen is expected to be present on MCF7 cells, as MCF7 is a breast cancer cell line. However, Ab3 generation was not much elevated when NLGP was used with Ab2 for immunization, than when Ab2 was used alone. The Ab3 generation in both cases was statistically notable. Ab3 also inhibited the binding between Ab1 and Ab2 as shown in Fig. 7. Completion of the idiotypic network was possible with BTAA and NLGP. Many set of experiments have earlier shown the generation of Ab3 with other antigen [19,20]

It is reasonable to state that it is possible to present the mirror image of BTAA, as Ab2, to generate an Ab3 response. This Ab3 could bind antigen, expressed on the cell surface, thereby, is expected to induce the Antibody-dependent cellular cytotoxicity. It is well known that if any antibody could induce Antibody-Dependent Cellular Cytotoxicity, it is expected to have a reasonable tumor-killing effect [21, 22]. Preclinical tests have to be performed if the idiotypic antibody could induce ADCC. Only, after good results in Preclinical tests, the question of testing the combination clinically will come. Though BTAA and NLGP could be considered a good candidate for therapeutic targets, it has to go a long way.

5. CONCLUSION

Anti-idiotypic antibodies have been found to have the ability to bring about humoral and cellular immune responses. This cellular and humoral immune response would help in killing the cells bearing the cancer antigens. Due to this, they could become potent cancer vaccines. The use of neem leaves for the generation of anti-idiotypic response would be safe as the non-toxicity of the neem leaves at this dose was shown earlier. In this way, this combination of BTAA and NLGP

should help in the development of an immunotherapeutic vaccine for patients, as this combination has been found to cause cellular and humoral immune response. However, it will be beneficial to the patient only if it shows good results in pre-clinical and clinical trials.

ACKNOWLEDGEMENTS

The first author recognizes the financial assistance provided by the Council of Scientific and Industrial Research through a CSIR-NET fellowship grant.

REFERENCES

[1] Baral, R., (2005) "Tumor vaccine: current trends in antigen specific immunotherapy", *Indian Journal ofExperimental Biology*, Vol. 4, No.5, pp389-406.

[2] Even-Desrumeaux, K., Baty, D., Chames, P., (2011)"State of the art in tumor antigen and biomarker discovery",*Cancers (Basel)*, Vol. 3, No. 2, pp2554-96. Doi: 10.3390/cancers3022554.

[3] Ronald, B. Herberman (1977) "Immunogenicity of tumor antigens",*Biochimica et BiophysicaActa (BBA)-Reviews on Cancer*, Vol. 473, No. 2, pp93-119. Doi.org/10.1016/0304-419X(77)90002-6.

[4] Meraviglia-Crivelli, D., Zheleva, A., Barainka, M., Moreno, B., Villanueva, H., Pastor, F., (2022) "Therapeutic Strategies to Enhance Tumor Antigenicity: Making the Tumor Detectable by the Immune System", *Biomedicines*, Vol. 10, No.8, pp1842. Doi: 10.3390/biomedicines10081842.

[5] Sichangi, M.Y., (2020) "Understanding Mechanisms of Actions for Vaccine Adjuvants Critical for Designing Effective Vaccines: A narrative review", *African Journal of Health Sciences*, Vol. 33, No. 3, pp18-29.

[6] Stanova, A.K., Ryabkova, V..A., Tillib, S.V., Utekhin, V.J., Churilov, L.P., Shoenfeld, Y., (2020) "Anti- Idiotypic Agonistic Antibodies: Candidates for the Role of Universal Remedy*",Antibodies*, Vol. 9, No. 2, pp19.Doi.org/ 10.3390/antib9020019

[7] Jerne, N.K., (1974) "Towards a network theory of the immune system", *Ann Immunol*, Vol. 125C, No. 1-2, pp373-89.

[8] Kohler, H., Pashov, A., Kieber-Emmons, T., (2019) "The promise of Anti-idiotype revisited", *Front. Immunol Sec. Vaccines and Molecular Therapeutics*; Vol. 10, No. 808, pp1-7.Doi.org/10.3389/fimmu.2019.00808.

[9] Foon, K.A., Sen, G., Hutchins, L., Kashala, O.L., Baral R., Banerjee, M., Chakraborty, M., Garrison, J., Reisfeld, R.A., Bhattacharya-Chatterjee, M., (1998) "Antibody responses in melanoma patients immunized with an anti- idiotype antibody mimicking disialoganglioside GD2", *Clinical cancer research: an official journal of the AmericanAssociation for Cancer Research.*, Vol. 4, No.5, pp1117-24.

[10] Samonigg, H., Wilders-Truschnig, M., Kuss, I., Plot, R., Stoger, H., Schmid. M., Bauernhofer, T., Tiran, A., Pieber, T., Havelec, L., Loibner, H., (1999) "A double-blind randomized-phase II trial comparing immunization with antiidiotype goat antibody vaccine SCV 106 versus unspecific goat antibodies in patients with metastatic colorectal cancer", Journal of Immunotherapy, Vol.22, No. 6, pp482-8.

[11] Reinartz, S., (2004) "Vaccination of Patients with Advanced Ovarian Carcinoma with the Anti-Idiotype ACA125: Immunological Response and Survival (Phase Ib/II)", *Clinical Cancer Research*, Vol. 10, No. 5, pp1580– 1587.Doi:10.1158/1078-0432.ccr-03-0056 .

[12] Saha, A., Chatterjee, S.K., (2010) "Dendritic cells pulsed with an anti-idiotype antibody mimicking Her-2/neu induced protective antitumor immunity in two lines of Her-2/neu transgenic mice", *Cellular Immunology*, Vol. 263, No. 1, pp 9-21. Doi.org/10.1016/j.cellimm.2010.02.010.

[13] Ladjemi, M.Z., (2012) "Anti-idiotypic antibodies as cancer vaccines: achievements and future improvements",*Frontiers in oncology*, Vol.2, No. 158, pp1-7.Doi.org/ 10.3389/fonc.2012.00158.

[14] Hutchins, L.F., Makhoul, I., Emanuel, P.D., Pennisi, A., Siegel, E.R., Jousheghany, F., Guo, X., Pashov, A.D., Monzavi-Karbassi, B., Kieber-Emmons, T., (2017) "Targeting tumor-associated carbohydrate antigens: a phase I study of a carbohydrate mimetic-peptide vaccine in stage IV breast cancer subjects", *Oncotarget*, Vol. 8, No. 58, pp99161-99178.DOI:1018632/oncotarget.21959.

[15] Makhoul, I, Hutchins, L, Emanuel, P.D., Pennisi, A., Siegel, E., Jousheghany, F., Monzavi-Karbassi, B., Kieber-Emmons, T., (2015) "Moving a carbohydrate mimetic peptide into the clinic: clinical response of a breast cancer patient after mimotope-based immunotherapy" *Human Vaccines*

&Immunotherapeutics, Vol. 11, No. 1, pp 37-44.Doi:10.4161/hv.34300.

[16] Naveed, A., Rahman, S.U., Arshad, M.I., Aslam, B., (2018) "Recapitulation of the anti-idiotypic antibodies as vaccine candidate", *Translational Medicine Communications*, Vol. 3, pp1-7.Doi.org/10.1186/s41231-018-0021.

[17] Pal, S., Sanyal, U,,Chattopadhyay, U., (1995) "Purification and characterization of a new 85-kDa glycoprotein antigen from human breast tumor", *International journal of cancer*, Vol. 60, No. 6, pp759-65.

[18] Mandal-Ghosh, I., Chattopadhyay, U., Baral, R.N., (2007) "Neem leaf preparation enhances Th1 type immune response and anti-tumor immunity against tumor associated antigen", *Cancer Immunity*, Vol. 7, pp 8-17.

[19] Bhattacharya-Chatterjee, M., Foon, K.A., Kohler, H.,(1991) "Anti-Idiotype Monoclonal Antibodies as Vaccinesfor Human Cancer" *International Reviews of Immunology*, Vol. 7,No.4,pp-289-302. Doi. org/10.3109/08830189109114876.

[20] Hernandez, A. M., Toledo, D., Martinez, D., Grinan, T., Brito, V., Macias, A., … Perez, R. (2008) "Characterization of the Antibody Response against NeuGcGM3 Ganglioside Elicited in Non-Small Cell Lung Cancer Patients Immunized with an Anti-Idiotype Antibody*" The Journal of Immunology,* Vol.181, No.9,pp6625– 6634.Doi:10.4049/jimmunol.181.9.

[21] Li, F & Liu, S, (2022) "Focusing on NK cells and ADCC: A promising immunotherapy approach in targeted therapy for HER2-positive breast cancer" *Front. Immunol.*Vol.13, No. 1083462, pp1-13.Doi: 10.3389/fimmu.2022.1083462.

[22] Boyerinas, B., Jochems, C., Fantini, M., Heery, C.R., Gulley, J.L., Tsang, K.Y., Schlom, J. , (2015)"Antibody- Dependent Cellular Cytotoxicity Activity of a Novel Anti-PD-L1 Antibody Avelumab (MSB0010718C) on Human Tumor Cells", *Cancer Immunol Res*, Vol. 3, No.10, pp1148-1157. Doi: 10.1158/2326-6066.CIR-15-0059.