An Immunoinformatics approach to predict T and B cell epitope of NY-BR-1 breast cancer antigen

A Thesis submitted in partial fulfillment of the requirements for the award of the degree of

MASTER OF SCIENCE IN BIOTECHNOLOGY

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Candidate's Declaration

I hereby declare that the work which is being presented in the dissertation entitled "An Immunoinformatics approach to predict T and B cell epitope of NY-BR-1 breast cancer antigen" in partial fulfillment of the requirements for the award of Master of Science in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala is an authentic record of my own work during a period of six months from January 2013 to June 2013, under the supervision of Dr. Manoj Baranwal, Assistant Professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala. The Report has not been submitted for the award of any other degree or certificate in this or any other university.

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This is to certify that the above statement given by the candidate is correct and true to the best of our knowledge.

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CERTIFICATE

This is to certify that the thesis entitled "**An Immunoinformatics approach to predict T and B cell epitope of NY-BR-1 breast cancer antigen"** being submitted by Avni Vij, Registration No. 301101007 in partial fulfillment of the requirements for the award of degree of Master of Science in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is a bonafide work carried out under my supervision and guidance. The thesis has not been submitted for award of any other degree or certificate in this or any other university.

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ABSTRACT

Breast cancer is one of the most common cancers in female around the world. Although many patients with breast cancer can be rendered free of disease with standard therapy such as surgery, radiation, and chemotherapy, some patients will have their disease recur. The identification of specific tumor antigens has significantly advanced in the field of tumor immunology, in particular, the development of cancer vaccines. The purpose of a vaccine is to induce an antigen-specific immune response that will result in disease prevention. Peptide based vaccines are one of the interesting strategies for developing vaccine against breast cancer. The use of synthetic peptides in vaccines offers practical advantages such as relative ease of construction and production, chemical stability, and a lack of infectious or oncogenic potential. T -cell epitopes were predicted from the consensus sequence of NY-BR-1 breast cancer antigen with the help of immunoinformatic tools for MHC Class I and Class II. Immunogenic peptides were generated by finding overlapping predicted epitopes. Twenty and six immunogenic peptides containing epitopes were identified for MHC Class I and II respectively. Finally we found two peptides which contained both MHC class I and class II T-cell epitopes. We have also conducted study for prediction of B-cell epitopes using immunoinformatic tools. Eleven immunogenic peptides containing B cell epitopes were finally selected. Interestingly, one immunogenic peptide was identified which contains MHC Class I restricted T-cell epitopes and a B-cell epitope. Immunogenic peptides which are finally selected based on the present study can be further evaluated to assess the immunogenic response and can become desirable vaccine candidates.

ABBREVIATIONS

ANN	Artificial Neural Network
APC	Antigen Presenting Cell
BIMAS	Bioinformatics and Molecular Analysis Section
CD	Cluster of Differentiation
CTL	Cytotoxic T Lymphocytes
DCIS	Ductal Carcinoma In situ
DMEM	Delbecco's Modified Essential Media
EDTA	Ethylene-diamine-tetraacetic acid
ELISA	Enzyme Linked Immunosorbant Assay
HIV	Human Immunodefeciency Virus
HLA	Human Leukocyte Antigen
HMM	Hidden Markov Models
IC	Inhibitory Concentration
IDC	Invasive ductal carcinoma
IEDB	Immune Epitope Data Base
ILC	Invasive lobular carcinoma
MSA	Multiple Sequence Alignment
MTT	3-(4, 5-dimethylthiazol-2-yl) – 2, 5-diphenyl tetrazolium bromide
NCBI	National Centre For Bioinformatic Information
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffer Saline
SVM	Support vector machine
TAA	Tumor-associated antigens
VLP	Virus like particles

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INTRODUCTION

Breast cancer is one of the most common cancers around the world. Breast cancer is a malignant tumor that starts in the cells of the breast. A malignant tumor is a group of cancer cells that can grow into (invade) surrounding tissues or spread (metastasize) to distant areas of the body. The disease occurs almost entirely in women, but men can get it, too.

In the US, breast cancer is the most common cancer amongst women and 1 in 8 women in the US have a chance of developing breast cancer in their life time. In India, the overall incidence of breast cancer is less as compared to the US. In the year 2008, there was about 1, 82,000 breast cancer cases reported in the US, whereas in India, 1, 15,000 new cases were diagnosed. This implies that, the percentage of total women in India's population was affected less as compared to US population. The breast cancer burden in India has almost reached about 2/3rds of that of the US and is steadily rising.

Many risk factors can increase the chance of developing breast cancer, but exact mechanism of how some of these risk factors cause cells to become cancerous is not clear. Vaccines have been one of the most successful clinical interventions for the prevention of human disease. The identification of specific tumor antigens has significantly advanced the field of tumor immunology, in particular, the development of breast cancer vaccines. Improved understanding of the molecular basis of antigen recognition has resulted in the development of rationally designed breast cancer antigen-specific vaccines based on prediction of epitope binding human class I or class II major histocompatibility molecules (MHC).

The use of synthetic peptides in breast cancer vaccines offers practical advantages such as relative ease of construction and production, chemical stability, and a lack of infectious or oncogenic potential. Peptides may also allow better manipulation of the immune response through the use of epitopes designed for stimulating particular subsets of T cells (e.g. helper and cytotoxic T cells). In animal models, peptide vaccines are particularly effective in generating immune responses to self-proteins (Disis *et al.,* 1996). Theoretically, immunization to foreign proteins normally elicits immunity to only a subset of potential epitopes, dominant epitopes, whereas other potentially immunogenic epitopes, subdominant epitopes, are ignored. Many newly defined tumor antigens are self-proteins; peptide immunization may play a key role in the ability to elicit an immune response to such antigens.

Peptide based vaccines can be designed to represent subdominant epitopes, thus, elicit immunity. The general idea behind the peptide vaccines is based on the chemical approach to synthesize the identified peptide containing B-cell and T-cell epitopes that are immunodominant and can induce specific immune responses. The first epitope-based vaccine was created in 1985 by Jackob *et al*. They introduced recombinant DNA expressing epitopes against cholera in Escherichia coli.

Immunoinformatics technologies have allowed for a more streamlined approach to vaccine design by identifying epitopes recognized across multiple alleles of major ethnic groups throughout the world (Kessler and Melief, 2007). Immunoinformatics is a relatively new field of immunomics, which focuses on the interactions between a pathogen and a host, erects a bridge across the endeavours of informatics, genomics, proteomics, immunology and clinical medicine.

Immunoinformatics research stresses mostly on the design and study of algorithms for mapping potential B- and T-cell epitopes, which speeds up the time and lowers the cost needed for laboratory analysis of pathogen gene products. Using such tools and information, an immunologist can analyse the sequence areas with potential binding sites, which in turn leads to the development of new vaccines. This is mainly beneficial because conventional methods need to dedicate time to pathogen cultivation and subsequent protein extraction. Although pathogens grow quickly, extraction of their proteins and then testing of those proteins on a large scale is expensive and time-consuming. Immunoinformatics is capable of reducing time and saving resources for the development of relevant vaccines by revealing virulence genes and surface-associated proteins.

Several classes of tumor antigens have been described in different cancer and NY-BR-1 is one interesting breast cancer antigen. NY-BR-1 represents an attractive target for cancer immunotherapy especially for vaccine design due its organ specificity (Theurillat *et al*., 2006).

Present study is focussed on the identification of peptide containing T and B cell epitope in NY-BR-1 breast cancer antigen using immunoinformatics tool.

2

REVIEW OF LITERATURE

The body is made up of trillions of living cells. Normal body cells grow, divide into new cells, and die in an orderly fashion. During the early years of a person's life, normal cells divide faster to allow the person to grow. After the person becomes an adult, most cells divide only to replace worn-out or dying cells or to repair injuries.

Cancer begins when cells in a part of the body start to grow out of control. There are many kinds of cancer, but they all start because of out-of-control growth of abnormal cells.

Cells become cancer cells because of damage to DNA. DNA is in every cell and directs all its actions. In a normal cell, when DNA gets damaged the cell either repairs the damage or the cell dies. In cancer cells, the damaged DNA is not repaired, but the cell does not die like it should. Instead, this cell goes on making new cells that the body does not need. These new cells will all have the same damaged DNA as the first cell does. (American Cancer Society, Breast Cancer Facts and Figures, 2011-2012)

Cancer cells often travel to other parts of the body, where they begin to grow and form new tumors that replace normal tissue. This process is called metastasis. It happens when the cancer cells get into the bloodstream or lymph vessels of our body.

No matter where a cancer may spread, it is always named for the place where it started like breast cancer that has spread to the liver but still called breast cancer, not liver cancer. Likewise, prostate cancer that has spread to the bone is metastatic prostate cancer, not bone cancer.

2.1 Breast cancer

Breast cancer is a malignant tumor that starts in the cells of the breast. A malignant tumor is a group of cancer cells that can grow into (invade) surrounding tissues or spread (metastasize) to distant areas of the body. The disease occurs almost entirely in women, but men can get it, too. (www.cancer.org/acs/groups/cid/documents/webcontent/003090-pdf.pdf)

2.1.1 General breast cancer terms

- **Carcinoma:** This is a term used to describe a cancer that begins in the lining layer (epithelial cells) of organs like the breast. Nearly all breast cancers are carcinomas (either ductal carcinomas or lobular carcinomas).

- **Adenocarcinoma:** An adenocarcinoma is a type of carcinoma that starts in glandular tissue (tissue that makes and secretes a substance). The ducts and lobules of the breast are glandular, so cancers starting in these areas are often called adenocarcinomas.
- **Carcinoma in situ:** This term is used for an early stage of cancer, when it is confined to the layer of cells where it began. In breast cancer, in situ means that the cancer cells remain confined to ducts (ductal carcinoma in situ). The cells have not grown into (invaded) deeper tissues in the breast or spread to other organs in the body. Carcinoma in situ of the breast is sometimes referred to as non-invasive or pre-invasive breast cancer because it might develop into an invasive breast cancer if left untreated. When cancer cells are confined to the lobules it is called lobular carcinoma in situ.
- **Invasive (infiltrating) carcinoma:** Invasive cancer is one that has already grown beyond the layer of cells where it started. Most breast cancers are invasive carcinomas—either invasive ductal carcinoma or invasive lobular carcinoma.
- **Sarcoma:** Sarcomas are cancers that start in connective tissues such as muscle tissue, fat tissue, or blood vessels. Sarcomas of the breast are rare.
- **Ductal carcinoma in situ:** Ductal carcinoma in situ (DCIS) is the most common type of non-invasive breast cancer. DCIS means that the cancer cells are inside the ducts but have not spread through the walls of the ducts into the surrounding breast tissue.
- **Invasive (or infiltrating) ductal carcinoma:** This is the most common type of breast cancer. Invasive (or infiltrating) ductal carcinoma (IDC) starts in a milk duct of the breast, breaks through the wall of the duct, and grows into the fatty tissue of the breast. At this point, it may be able to spread (metastasize) to other parts of the body through the lymphatic system and bloodstream. About 8 of 10 invasive breast cancers are infiltrating ductal carcinomas.
- **Invasive (or infiltrating) lobular carcinoma:** Invasive lobular carcinoma (ILC) starts in the milk-producing glands (lobules). Like IDC, it can spread (metastasize) to other parts of the body. About 1 invasive breast cancer in 10 is an ILC. Invasive lobular carcinoma may be harder to detect by a mammogram than invasive ductal carcinoma.

2.1.2 Less common types of breast cancer:

- **Inflammatory breast cancer:** This uncommon type of invasive breast cancer accounts for about 1% to 3% of all breast cancers. Usually there is no single lump or tumor.

- **Triple-negative breast cancer:** This term is used to describe breast cancers (usually invasive ductal carcinomas) whose cells lack estrogen receptors and progesterone receptors, and do not have an excess of the HER2 protein on their surfaces. Triple-negative breast cancers tend to grow and spread more quickly than most other types of breast cancer.
- **Phyllodes tumor:** This very rare breast tumor develops in the stroma (connective tissue) of the breast, in contrast to carcinomas, which develop in the ducts or lobules. Other names for these tumors include phylloides tumor and cystosarcoma phyllodes. These tumors are usually benign but on rare occasions may be malignant. Benign phyllodes tumors are treated by removing the tumor along with a margin of normal breast tissue.
- **Sarcoma:** Adult Soft Tissue Cancer.
- **Angiosarcoma:** This form of cancer starts in cells that line blood vessels or lymph vessels. It rarely occurs in the breasts.

2.1.3 Special types of Invasive breast carcinoma

There are some special types of breast cancer that are sub-types of invasive carcinoma.

These include:

- Adenoid cystic carcinoma
- Low-grade adenosquamous carcinoma.
- Medullary carcinoma
- Mucinous carcinoma
- Papillary carcinoma
- Tubular carcinoma
- Metaplastic carcinoma
- Micropapillary carcinoma
- Mixed carcinoma (has features of both invasive ductal and lobular)

2.2 Causes of breast cancer

The causes of breast cancer are unknown. But certain things called risk factors can increase a woman's chances of getting breast cancer. Some women do seem to be at a higher risk of developing the disease.

- **Age:** The risk of developing breast cancer increases with age. It is rare in women under 35. 8 out of 10 breast cancers (80%) occur in women aged 50 or over.
- **Previous cancer and other breast conditions:** Women who had breast cancer or other breast conditions in the past may be at a higher risk of developing breast cancer. This includes women who have previously had:
    - Breast cancer, including ductal carcinoma in situ
    - Lobular carcinoma in situ
    - An over-production of slightly abnormal cells called atypical ductal hyperplasia
    - Radiotherapy to the chest to treat Hodgkin lymphoma at a young age
    - Dense breast tissue (when the breast is made up of glandular and connective tissue with very little fatty tissue).
- **Hormonal factors:** Exposure to the hormones oestrogen and progesterone for long, uninterrupted periods can affect your breast cancer risk.
- **Genetic factors (family history) -** 5−10% of breast cancers are thought to be linked to an inherited breast cancer gene. The genes most commonly linked to an increased risk of breast cancer in families are BRCA1 and BRCA2.

2.3 Symptoms of breast cancer

Symptoms of breast cancer are:

- A lump in the breast
- A change in the size or shape of the breast
- Dimpling of the skin or thickening in the breast tissue
- A nipple that is turned in (inverted)
- A rash (like eczema) on the nipple
- Discharge from the nipple
- Swelling or a lump in the armpit

2.4 Treatment

2.4.1 Oncoplastic surgery

Breast-conserving surgery (lumpectomy or partial mastectomy) can often be used for early-stage breast cancers. But in some women, it can result in breasts of different sizes and/or shapes. Breast-conserving surgery might not even be possible for larger tumors, hence a mastectomy might be needed. Some doctors address this problem by combining cancer surgery and plastic surgery techniques, known as oncoplastic surgery. This typically involves reshaping the breast at the time of the initial surgery, and may mean operating on the other breast as well to make them more symmetrical. This approach is still fairly new, and not all doctors are comfortable with it.

2.4.2 Breast reconstruction surgery

The number of women with breast cancer choosing breast conservation therapy has been steadily increasing, but there are some women who, for medical or personal reasons, choose mastectomy. Concern over a possible link between breast implants and immune system diseases has discouraged for several years. Recent studies have found that although implants can cause some side effects (such as firm or hard scar tissue formation), women with implants do not have any greater risk for immune system diseases than women who have not had this surgery. Similarly, the concern that breast implants increase the risk of breast cancer recurrence or formation of new cancers is not supported by current evidence.

2.4.3 Radiation therapy

Women who need radiation after breast-conserving surgery, newer techniques such as hypofractionated radiation or accelerated partial breast irradiation may be as effective as opposed to the standard daily radiation treatments that take several weeks to complete. These techniques are being studied to see if they are as effective as standard radiation in helping prevent cancer recurrences.

2.4.4 New chemotherapy drugs

Advanced breast cancers are often hard to treat, so researchers are always looking for newer drugs. A drug class has been developed that targets cancers caused by BRCA mutations. This class of drugs is called PARP inhibitors and they have shown promise in clinical trials treating breast, ovarian, and prostate cancers that had spread and were resistant to other treatments. Further studies are being done to see if this drug can help patients without BRCA mutations.

2.4.5 Targeted therapies

Targeted therapies are a group of newer drugs that specifically take advantage of gene changes in cells that cause cancer.

- **Drugs that target HER2:** Recently, a new drug for patients whose cancer cells have too much HER2 protein has been approved by the FDA. This drug, ado-trastuzumab emtansine (Kadcyla™) was formerly called TDM-1. It is made up of the same monoclonal antibody found in trastuzumab (Herceptin) attached to a chemotherapy drug known as DM-1. This drug is given as an injection into a vein (IV) every 3 weeks. Common side effects include fatigue, nausea, muscle and bone pain, low platelet counts, headache, and constipation. This drug can also cause more serious side effects, such as severe allergic reactions, liver damage, heart damage, and lung problems.
- **Anti-angiogenesis drugs:** Blood vessels must develop to nourish the cancer cells. This process is called angiogenesis. `Bevacizumab (Avastin) is an example of anti-angiogenesis drug. Although bevacizumab turned out to not be very helpful in the treatment of breast cancer, this approach still may prove useful in breast cancer treatment.
- **Other targeted drugs:** Everolimus (Afinitor) is a targeted therapy drug that seems to help hormone therapy drugs work better. It is approved to be given with exemestane (Aromasin) to treat advanced hormone receptor-positive breast cancer in postmenopausal women Other potential targets for new breast cancer drugs have been identified in recent years. Drugs based on these targets are now being studied, but most are still in the early stages of clinical trials.
- **Bisphosphonates :** Bisphosphonates are drugs that are used to help strengthen and reduce the risk of fractures in bones that have been weakened by metastatic breast cancer. Examples include pamidronate (Aredia) and zoledronic acid (Zometa).
- **Denosumab :** Denosumab (Xgeva, Prolia) can also be used to help strengthen and reduce the risk of fractures in bones that have been weakened by metastatic breast cancer.
- **Vitamin D:** A recent study found that women with early-stage breast cancer who were vitamin D deficient were more likely to have their cancer recur in a distant part of the body and had a poorer outlook. More research is needed to confirm this finding, and it is not yet clear if taking vitamin D supplements would be helpful.

2.5 Breast cancer and Immune system

The immune system plays a dual role in breast cancer, both promoting tumorigenesis through inflammatory pathways and preventing tumor formation through active immune monitoring. Along with this, some breast cancer patients display clear evidence of immune suppression. They have lower absolute numbers of lymphocytes (Caras *et al*., 2004) and Vα24Vβ1 natural killer T (NKT) cells in the peripheral blood (Molling *et al*., 2005), with decreased STAT1 signaling and IFN-γ production in both lymphocytes and NK cells (Konjevic *et al*., 2011). $CD3^+CD8^+$ cytotoxic T cells derived from breast cancer patients display a significant down regulation of the T-cell receptor (TCR)-ζ chain and cell surface CD28, and a significant up regulation of CD95 (FAS) (Gruber *et al.*, 2008). Patients with breast cancer have increased numbers of both $CD4^+CD25^+$ regulatory T cells (Tregs) (Wolf *et al*., 2003) and myeloid-derived suppressor cells (MDSC) (Diaz-Montero *et al.*, 2009) within the peripheral blood, and elevated serum arginase levels (Polat *et al*., 2003). Dendritic cells (DCs) within the peripheral blood of breast cancer patients are diminished in number and are dysfunctional, with low cell-surface MHC Class II and B7 (CD86) moleculesn (Pockaj *et al.*, 2004) and lower IL-12 secretion (Pinzon-Charry *et al*., 2007). Notably, the presence of immune cells in breast cancers also exerts an anti-tumor effect, and produces a favourable response to cancer therapy. DCs, M1 macrophages, Th1 $CD4^+$ T cells, cytotoxic $CD8^+$ T cells and NK cells protect against tumor growth, whereas M2 macrophages, MDSCs, neutrophils, Th2 $CD4^+$, Th17 $CD4^+$ and $FoxP3^+$ $CD4^+$ T cells promote tumor growth (Fridman *et al*., 2011).

2.6 Vaccines

The word ‘vaccination’ was used for first time by Edward Jenner in 1796 to describe the injection of smallpox vaccine. Louis Pasteur developed the concept through his innovative work in microbiology. Now, vaccination is the administration of antigenic agents applied to stimulate the immune system of an individual and to develop adaptive immunity to a disease. Vaccines can improve, or often even prevent, the effects of infection. Vaccination is generally considered to be the most effective method of preventing infectious diseases, and the efficacy of vaccination has been extensively studied and verified. The administration of some vaccines is conducted after the patient has already been infected by the pathogen. There are numerous vaccine examples, including experimental ones against AIDS, cancer and Alzheimer’s disease. The core mechanism behind all the vaccinations is the ability of the vaccine to initiate an immune response in a

quicker fashion than the pathogen itself. The purpose of every vaccination is to present a particular antigen or set of antigens to the immune system in order to evoke a relevant immune response. The main active component of a vaccine may be inactive, but still intact (attenuated bacteria or viruses), or purified components of the pathogen that are known to induce immune reaction. There are different types of vaccines such as live attenuated vaccines, inactivated vaccines, subunit vaccines, peptide vaccines, DNA vaccines etc.

2.6.1 Peptide vaccines

The improved knowledge of antigen recognition at molecular level has contributed to the development of rationally designed peptide vaccines. The general concept behind the peptide vaccines is based on the chemical approach to synthesize the identified B-cell and T-cell epitopes that are immunodominant and can induce specific immune responses. B-cell epitope of a target molecule can be conjugated with a T-cell epitope to make it immunogenic. The first epitope-based vaccine was created in 1985 by Jackob *et al*. They introduced recombinant DNA and expressed epitopes against cholera in Escherichia coli. Thus, Epitope-based vaccines can be constructed for T and B lymphocytes. The T-cell epitopes are typically peptide fragments, whereas the B-cell epitopes can be proteins, lipids, nucleic acids or carbohydrates. Peptides have become desirable vaccine candidates due to their comparatively easy production and construction, chemical stability, and absence of infectious potential. The peptide vaccines against various cancers have been developed, and entered phase I and phase II of clinical trials, with satisfactory clinical outcome as listed in Table 1. The peptide vaccination is commonly being studied for application in both ameliorating and prophylactic immunotherapy (Naz and Dabir, 2007). Yet there is more to be improved in order to eliminate obstacles, such as the need for a better adjuvant and carrier or the low immunogenicity.

TABLE 1: Peptide based vaccination trials in cancer patients.

Tumor	Peptide vaccine	Trial phase	Reference
Melanoma	gp100(g209)-2M	II	Rosenberg *et al*., 1998
	MART-127–35	I	Cormier et *al*., 1997
	gp100	I/II	Rosenberg *et al*., 1999
	MART-127–35	I/II	Wang *et al*., 1999
	gp100 (210M) + tyrosinase	II	Lee *et al*., 2001
	MAGE-3.A1	I/II	Marchand *et al*., 1999
	Tyrosinase	II	Scheibenbogen *et al*., 2000
Melanoma and others	NY-ESO-1	I/II	Jager *et al*.,2000
Pancreatic cancer	K-Ras/12	I/II	Gjertsen *et al*., 2001
CIN	HPV-16/E7 + KSS/PADRE	I/II	Muderspach *et al*., 2000
MART-1 = melanoma antigen recognized by T cells;MAGE-3.A1 = melanoma antigen-3 peptide restricted by HLA-A1;NY-ESO-1 = New York-eso phagus antigen-1;K-Ras/12 = K-Ras mutated at position 12;CIN = cervical intraepithelial neoplasia; HPV = human papilloma virus; E7 = early protein 7; KSS = amino acid sequence of the linker peptide;PADRE= pan-DR epitope.			

2.7 Immunoinformatics

The accelerating growth of bioinformatics techniques and applications along with the substantial amount of experimental data has made a significant impact on the immunology research. This has led to a rapid growth in the field of computational immunology, and a number of immunology-focused resources and software, which help in understanding the properties of the whole immune system, have become available. This has given rise to a new field, called immunoinformatics. Immunoinformatics can be described as a branch of bioinformatics concerned with in silico analysis and modelling of immunological data and problems. Immunoinformatics research stresses mostly on the design and study of algorithms for mapping potential B- and T-cell epitopes, which speeds up the time and lowers the cost needed for laboratory analysis of pathogen gene products. Using such tools and information, an immunologist can analyse the sequence areas with potential binding sites, which in turn leads to

the development of new vaccines. The methodology of analysing the pathogen genome to identify potential antigenic proteins is known as 'reverse vaccinology'. This is mainly beneficial because conventional methods need to dedicate time to pathogen cultivation and subsequent protein extraction. Although pathogens grow quickly, extraction of their proteins and then testing of those proteins on a large scale is expensive and time-consuming. Immunoinformatics is capable of reducing time and saving resources for the development of relevant vaccines by revealing virulence genes and surface-associated proteins. Normally, the investigation of the binding affinity of antigenic peptides to the MHC molecules is the main goal when predicting epitopes. The experimental techniques are found to be difficult and time-consuming, and therefore several in silico methodologies are being created and constantly improved to identify epitopes. The list of approaches includes matrix-driven methods, QSAR analysis, identification of structural binding motifs, protein threading, homology modelling, docking techniques, and design of several machine-learning algorithms and tools (Tomar and RK, 2010). In the past, computational techniques could only identify sequence characteristics, but new improved algorithms and tools are being designed to increase the predictive performance. The methods used for development of prediction models can be divided into structure-based methods that derive information from the three-dimensional structure of the proteins, and sequence-based methods that analyse the amino acid sequence.

2.8 T-cell and B-cell epitopes and their prediction algorithms

The epitope is recognizable by the immune system part of the antigen, and in particular by antibodies, B cells or T cells. The epitopes may belong to both foreign and self proteins, and they can be categorized as conformational or linear, depending on their structure and integration with the paratope. T-cell epitopes are presented on the surface of an antigen presenting cell (APC), where they are bound to major histocompatibility (MHC) molecules in order to induce immune response. MHC class II proteins bind oligopeptide fragments derived through the proteolysis of pathogen antigens, and present them at the cell surface for recognition by CD4+ T cells. If sufficient quantities of the epitope are presented, the T cell may trigger an adaptive immune response specific for the pathogen. Class II MHCs are expressed on specialized cell types, including professional APCs such as B cells, macrophages and dendritic cells, whereas class I MHCs are found on every nucleated cell of the body. The MHC I molecule binds to a peptide of approximate 9 amino acids in length within a closed groove. In contrast, because the antigen-binding groove is open at both ends, the MHC II molecules can present much longer peptides,

generally varying from 12 to 25 amino acids, nine of which occupy the binding groove. This difference between MHC I and MHC II is very important for the development of distinct prediction algorithms (Larson *et al*., 2006).

The recognition of epitopes by T cells and the induction of immune response have a key role for the individual's immune system. Even the slightest deviation from the normal functioning can have a grave impact on the organism. In case of autoimmune disease, the T cells recognize the cells' native peptides as foreign, and attack and eventually destroy the organism's own tissues. Some viruses, such as human immunodeficiency virus (HIV), hepatitis C, and avian and swine influenza, manage to avoid recognition by the T cell relying on various mutations that effectively alter the amino acid sequences of the proteins encoded by the viral genes. Knowledge about the peptide's epitopes has a key role for developing epitope-based vaccines, which, injected into the recipient, can induce immune response (Anderson *et al*., 2006). One of the key issues in T-cell epitope prediction is the prediction of MHC binding, as it is considered a prerequisite for T cell recognition. All T-cell epitopes are good MHC binders, but not all good MHC binders are T-cell epitopes. Determining the peptide binding preferences exhibited by this extensive set of alleles is beyond the present capacity of experimental techniques, necessitating the development of bioinformatics prediction methodologies. The most successful prediction methods for T-cell epitopes developed to date have been data-driven. T-cell epitope prediction typically involves defining the peptide binding specificity of specific class I or class II MHC alleles and then predicting epitopes in silico. Using peptide sequence data, experimentally determined affinity data have been used in the construction of many T-cell epitope prediction algorithms. Such methods include motif-based systems, support vector machines (SVMs), and hidden Markov models (HMMs), quantitative structure–activity relationship (QSAR) analysis and structure-based approaches.

Compared to T cell epitopes prediction algorithms, the B cell epitope prediction is more complicated, especially for the conformational B cell epitopes because, in addition to the sequence composition, the 3D-structure of protein must also be considered. The development of B cell epitopes prediction algorithms has been less successful compared to T cell epitope prediction, especially in accuracy (Anderson *et al*., 2006). There are several reasons for this. For instance, the majority of B cell epitopes are discontinuous so that it is hard to determine the relevant amino acids and the distribution of the antigen surface. Moreover, much of the experimental data based on the prediction algorithms are still controversial because of the poorly understood recognition properties of cross reactive antibodies (Davies *et al*., 2007).

Nevertheless, in spite of these difficulties, there are several methods available for B cell epitope prediction for both linear and conformational epitopes. The prediction algorithms for linear B cell epitopes are similar to the T cell's. Similarly, the accuracy of primary sequence-based algorithms is low (Jameson *et al*., 1988), and modified algorithms based on machine learning were subsequently developed, such as ABCpred (Saha et. al., 2006) and BepiPred (Larsen *et al*., 2006) with significant improvements in accuracy. Prediction algorithms for conformational B cell epitopes based on 3D structure are also available owing to the ever-increasing 3D structure of antigen-antibody complex data. Some prediction servers based on this algorithm are accessible, for example DiscoTope and CEP (http:// bioinfo.ernet.in/cep.htm) (Kulkarni *et al*., 2005). These methods make use of information carried in the structure of antibodies against proteins of interest to reveal the 3D folding of target proteins.

2.9 NY-BR-1: Target for vaccine design

The NYBR-1 gene is located on chromosome 10p11-p12 and is composed of 37 exons. It encodes a peptide of M_r 150,000– 160,000, a putative transcription factor. Since its protein expression is restricted to normal and neoplastic breast epithelium, NY-BR-1 is classified as a differentiation antigen of the mammary gland. A recent immunohistochemical study with a limited number of breast carcinoma lesions has shown that NY-BR-1 is expressed in about 60% of breast carcinoma and has confirmed the breast epithelium specificity.

NY-BR-1 represents an attractive target for cancer immunotherapy and can be potentially used for diagnostic purposes in surgical pathology as it is present in breast cancer metastasis indicating that its expression is not lost during disease progression (Jager *et al*., 2005). It also shows breast epithelium Specificity (Theurillat *et* al., 2007). It is also expressed at the cell membrane of tumor cells *in vitro* as well as *in vivo*. NY-BR-1 localizes to the cytoplasm and the cell membrane. Its highly restricted expression of NY-BR-1 in normal breast and breast cancer also makes it an attractive target for designing a vaccine against breast cancer (Gure *et al*., 2001). There also exists strong co-expression of both HLA Class I and NY-BR-1 antigen which is a very essential requirement for stimulating immune system (Theurillat *et al*., 2007). Many studies prove that its expression is not influenced by preceding chemotherapy.

Like several other differentiation antigens, NY-BR-1 has a lower expression in metastatic lesions, because tumors of higher histological grade are more likely to metastasize. Specifically, NY-BR-1 expression decreases in the following order: carcinoma in situ (CIS) > invasive

carcinoma > lymph node metastasis > distant metastasis (Gure *et al*., 2001). NYBR- 1 is present in a large number of tumors without EGFR/ HER2 expression, suggesting that NY-BR-1 immunotherapy is a potential treatment option for patients with such tumors. Active specific immunotherapy is mostly applied in patients with metastatic disease, who have already received chemotherapy in an adjuvant or rarely in a neoadjuvant setting. Therefore it is important to know whether chemotherapy could influence NY-BR-1 expression. Studies indicate that neoadjuvant chemotherapy may have no effect on NY-BR-1 expression, suggesting that active specific immunotherapy targeting NY-BR-1 can be considered even in patients who have been treated with several chemotherapy cycles (Theurillat *et al*., 2007). Thus, NY-BR-1 meets most, if not all the criteria required to be utilized as a target for T-cell-based immunotherapy.

3

OBJECTIVE

The main objective of the present study carried out was to predict T and B-cell epitopes of breast cancer antigen NY-BR-1 which can be used as a vaccine against breast cancer.

Work plan of the current study is as follows:

1. Finding out the consensus sequence, from all available sequences of NY-BR-1 breast cancer antigen.

2. Prediction of T and B-cell epitope in the consensus sequence of the antigen using different immunoinformatics tools.

3. Optimization of MTT assay to estimate cell proliferation of peripheral blood mononuclear cells.

4

MATERIAL AND METHODS

4. 1 Consensus sequence of NY-BR-1

4.1.1 Sequence Retrieval

The protein sequences of NY-BR-1 were taken from protein database of NCBI (http://www.ncbi.nlm.nih.gov/protein/NY-BR-1). All sequences which were from human source were collected. The synthetic constructs were ignored. Three related sequences were obtained which were saved in FASTA format.

4.1.2 Multiple Sequence Alignment

Multiple sequence alignment (MSA) is a basic tool for sequence analysis. It is carried out to identify highly conserved or similarity regions within a set of related sequences. Multiple sequence alignment was carried out by CLUSTAL W tool.

CLUSTAL W (http://www.ebi.ac.uk/clustalw/)

CLUSTAL W is a general purpose Multiple Sequence Alignment program for DNA and proteins. It produces biologically meaningful multiple sequence alignments of divergent sequences. It calculates the best match for the selected sequences, and lines them up so that the identities, similarities and differences can been seen. Evolutionary relationships can also be seen via viewing cladograms or phylograms. The results of the multiple sequence alignment by CLUSTAL W were saved in FASTA format.

4.1.3 Consensus Sequence

Consensus sequence was found out from the results of multiple sequence alignment using the jalview function of CLUSTAL W tool. The consensus sequence so obtained was copied and pasted in a separate word document.

4.2 T-Cell Epitope Prediction

4.2.1 MHC Class I Epitope Prediction

There are different immunoinformatics tools for epitope prediction but only one of them was optimized and used to identify MHC class I epitopes.

BIMAS (http://www.bimas.cit.nih.gov/molbio/hla_blind/)

BIMAS stands for Bioinformatics and Molecular Analysis Section. BIMAS locate and rank 8-mer, 9-mer, or 10-mer peptides that contain peptide-binding motifs for HLA class I molecules. BIMAS works on the Principle of Quantitative matrices. In this method, the contribution to binding from each amino acid at each peptide position within the binding groove is quantified (Parker *et al.*, 1994). It is assumed that each position within the peptide contributes independently in binding to an MHC molecule, and a residue located at a given peptide position contributes an equal amount to binding, even within different peptides. This method involves producing a matrix in which every entry (X, Y) represents a score associated with amino acid residue X at position Y. The position-specific amino acid values reflect the structural properties of HLA alleles, therefore representing a fingerprint for HLA binding domains.

Consensus sequence obtained earlier was taken as the input in FASTA format and analysis was carried out for HLA class I molecules at different parameters. Score was selected in the form of $T_{(1/2)}$ (estimate of half time of dissociation of a molecule containing this subsequence).

Different parameters were set as follows:

1. Length of the epitope = 9 amino acids (nonamer)

2. Predicted $T_{(1/2)} \geq$ 1, 5, 10, 20, 50, 100, 200, 500 for different MHC alleles.

Epitopes having score equal to or more than threshold value as well as predicted to bind one or more number of HLA Alleles were taken in account.

4.2.2 MHC Class II Epitope Prediction

Many tools are available for class II MHC binding epitope but in our study we have used NetMHCII 2.2 server (http://www.cbs.dtu.dk/services/NetMHCII/).

NetMHCII 2.2 server predicts binding of peptides to HLA-DR, HLA-DQ, HLA-DP MHC class II alleles using artificial neural network. The artificial neural network method includes explicit encoding of the peptide flanking residues in terms of amino acid composition and length, as well as a novel scheme for neural network training that deals with the data redundancy inherent in the peptide data due to multiple examples of identical binding cores (Neilsen *et al.*, 2009). The prediction values are given in nM IC_{50} values, and as a percentage rank to a set of 1,000,000 random natural peptides. Strong and weak binding peptides are indicated in the output. The accuracy of the peptide binding core identification has been improved using a neural network alignment procedure. This has made obsolete the need for P1 amino acids encoding.

Consensus sequence was taken as the input in FASTA format and analysis was carried out for all HLA class II molecules, turning off PI amino acid preference. Strong Binders ($IC_{50} \leq 50$) were taken into consideration.

4.3 B-Cell Epitope Prediction

Compared to T cell epitopes prediction algorithms, the B cell epitope prediction is more complicated, especially for the conformational B cell epitopes because, in addition to the sequence composition, the 3D-structure of protein must also be considered.

IEDB Analysis Resource (**(**http://tools.immuneepitope.org/tools/bcell/iedb_input) was used for predicting B-Cell epitopes. IEDB provides different B cell prediction methods:

- Chou and Fasman beta turn prediction
- Emini surface accessibility scale
- Karplus and Schulz flexibility scale
- Kolaskar and Tongaonkar antigenicity scale
- Parker Hydrophilicity Prediction
- Bepipred Linear Epitope Prediction

Parameters such as hydrophilicity, flexibility, accessibility, turns, exposed surface, polarity and antigenic propensity of polypeptides chains have been correlated with the location of continuous epitopes. This has led to a search for empirical rules that would allow the position of continuous epitopes to be predicted from certain features of the protein sequence. All prediction calculations are based on propensity scales for each of the 20 amino acids. Each scale consists of 20 values assigned to each of the amino acid residues on the basis of their relative propensity to possess the property described by the scale. Greater than 1.0 indicates an increased probability for being found on the surface. Only two methods were used to predict the B-cell epitopes.

- **Kolaskar and Tongaonkar antigenicity scale :** A semi-empirical method which makes use of physicochemical properties of amino acid residues and their frequencies of occurrence in experimentally known segmental epitopes was developed to predict antigenic determinants on proteins. Application of this method to a large number of proteins has shown by the authors that the method can predict antigenic determinants with about 75% accuracy which is better than most of the known methods.

- **Bepipred Linear Epitope Prediction:** BepiPred predicts the location of linear B-cell epitopes using a combination of a hidden Markov model and a propensity scale method.

Steps involved were:

- Entered protein sequence in plain format
- Selected a prediction method
- Submission

4.4 Optimization of MTT assay to estimate the cell proliferation

TABLE 2: Materials required for MTT assay.

Sr. No.	REQUIREMENTS	COMPANY
1.	Powdered RPMI Media	Himedia
2.	Sodium bicarbonate	Himedia
3.	L-Gluamine	Himedia
4.	Foetal Bovine Serum	Sigma Aldrich
5.	Penicillin Sodium	Himedia
6.	Streptomycin sulphate	Himedia
7.	Amphotericin	Himedia
8.	Hisep LSM 1073	Himedia
9.	Trypan Blue	Himedia
10.	MTT	Sigma Aldrich
11.	Dimethyl Sulfoxide	SRL
12.	ConA	Sigma Aldrich
13.	Sodium chloride	Himedia
14.	Potassium chloride	Himedia
15.	Sodium hydrogen phosphate	Himedia
16.	Potassium dihydrogen phosphate	Himedia

4.4.1 PREPARATION OF POWDERED RPMI MEDIA

10.3 g of powder RPMI media was suspended in 900 ml distilled water and constantly, stirred gently until the powder was completely dissolved and autoclaved for 15 minutes at 121□ C and 15 lps pressure in an autoclave. After autoclaving allow it to cool to room temperature and then add 26.7 ml of 7.5% sodium bicarbonate solution and 10.3 ml of 200 mM L-glutamine solution to 1 liter of medium and stirred until dissolved. pH was adjusted to 4.0 using 1N HCl or 1N NaOH pH of the medium was adjusted ±0.2 below the desired pH since the pH tends to rise

during filtration. The final volume was made up to 1000 ml with double distilled water. The medium was immediately sterilized by filtering through a sterile membrane filter with porosity of 0.22 micron or less, using positive pressure rather than vacuum to minimize the loss of carbon dioxide. Liquid medium was stored at 2-8° C and in dark till use. 10% heat inactivated fetal bovine serum (57° C for 30 minutes) and filter sterilized antibiotics [Streptomycin (100µg/ml), Penicillin (100 IU/ml), Amphotericin (2.5 µg/ml)] were added to media before culturing of cells.

4.4.2 Preparation of PBS

One litre of 1X PBS was prepared by adding 8 g of NaCl, 0.2 g of KCl, 1.44g of Na_2HPO_4. 0.24 g of KH_2PO_4 was added in 800 ml of distilled water. pH was adjusted to 7.4 using HCl (0.1N) and NaOH (0.1N). Volume was made up to 1 litre by distilled water. PBS was autoclaved for 20 minutes at 121 ^{0}C. After autoclaving PBS was stored at 4^0 C temperature.

4.4.3 Isolation of Peripheral Blood Mononuclear Cells

Blood was drawn from a healthy person with the help of vacutainer system (EDTA coated, Becton Dickinson). Blood was diluted in 1:1 ratio with PBS. Then blood sample was layered carefully over Hisep LSM 1073 in the ratio 4:3 and it was centrifuged at 700xg for 40 minutes at 25^0 C. Plasma was removed and then the Buffy coat layer was transferred to a clean centrifuge tube with the help of micropipette. Buffy coat layer was washed twice with 3 volumes of PBS by centrifuging at 1000 rpm for 12 minutes at 25^0C. Supernatant was discarded and pellet of PBMC was suspended in 1 ml of cell culture medium [RPMI + 10% FBS + Penicillin (100units/ml) + Streptomycin (100 µg/ml)].

4.4.4 Cell Counting and Viability testing

Cell counting was done with the help of hemocytometer using a trypan blue as a stain. Trypan blue is a stain which penetrates cell membrane of dead cells and dead cells are stained blue while live cells remain unstained. 20 µl of cell suspension was mixed with 20 µl of 0.4% trypan blue. The dilution was incubated for three to five minutes at room temperature. 10 µl of the trypan blue/ cell mixture was then injected beneath the cover slip on hemocytometer. The hemocytometer was placed on the stage and focused on using the 10X objective of the microscope and the cells were counted in all 4 sets of squares of haemocytometer using 40X objective of the microscope. Cell count and percent cell viability was calculated using following formula:

Cell count = [Total number of cells counted] x Dilution factor x 10^4
[Number of chambers counted]

Percent Cell Viability = [Total number of cells counted] x 100
[Total cells counted]

4.4.5 PBMC proliferation and MTT Assay

MTT assay is a calorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent and the released, solubilised formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is the measure of the viability of the cells. Therefore cell proliferation was tested using MTT assay.

For this assay, freshly isolated lymphocytes ($10x10^5$ cells to $1x10^5$ cells /200 µl media per well) were seeded in 96-well flat bottom microtiter plate taking Concanavalin-A (5-10 µg/ml) as positive control (ConA is a plant mitogen which induces mitosis in human T-lymphocytes and hence leads to T-cell proliferation). Plate was incubated at 37^0 C and 5% CO_2 concentration for 5 days. After 5 days, 10µl of MTT (5 mg/ml) was added to each well and an incubation of another 4 hours was given for reduction of MTT to formazan. Media was removed carefully and purple formazan crystals were dissolved in 100µl of DMSO. Absorbance was recorded at 570 nm by microtiter plate reader (Thermo Scientific).

5
Results And Discussion

5.1 Consensus Sequence of NY-BR-1 antigen

Three complete sequences of NY-BR-1 antigen were found in protein database of NCBI. These sequences were aligned using CLUSTAL W to find out the consensus sequence. The consensus sequence of NY-BR-1 antigen was 1341 amino acid long (TABLE 3). This consensus sequence was considered for further prediction of T-cell as well as B-Cell epitopes.

TABLE 3: Consensus sequence of NY-BR-1 antigen

MTKRKKTINLNIQDAQKRTALHWACVNGHEEVVTFLVDRKCQLDVLDGEHRTPLMKALQCHQEACANILIDSGADIN
LVDVYGNTALHYAVYSEILSVVAKLLSHGAVIEVHNKASLTPLLLSITKRSEQIVEFLLIKNANANAVNKYKCTALMLAVC
HGSSEIVGMLLQQNVDVFAADICGVTAEHYAVTCGFHHIHEQIMEYIRKLSKNHQNTNPEGTSAGTPDEAAPLAERT
PDTAESLVEKTPDEAAPLVERTPDTAESLVEKTPDEAASLVEGTSDKIQCLEKATSGKFEQSAEETPREITSPAKETSEKFT
WPAKGRPRKIAWEKKEDTPREIMSPAKETSEKFTWAAKGRPRKIAWEKKETPVKTGCVARVTSNKTKVLEKGRSKMI
ACPTKESSTKASANDQRFPSESKQEEDEEYSCDSRSLFESSAKIQVCIPESIYQKVMEINREVEEPPKKPSAFKPAIEMQN
SVPNKAFELKNEQTLRADPMFPPESKQKDYEENSWDSESLCETVSQKDVCLPKATHQKEIDKINGKLEESPNKDGLLK
ATCGMKVSIPTKALELKDMQTFKAEPPGKPSAFEPATEMQKSVPNKALELKNEQTLRADEILPSESKQKDYEENSWDT
ESLCETVSQKDVCLPKAAHQKEIDKINGKLEGSPVKDGLLKANCGMKVSIPTKALELMDMQTFKAEPPEKPSAFEPAIE
MQKSVPNKALELKNEQTLRADEILPSESKQKDYEESSWDSESLCETVSQKDVCLPKATHQKEIDKINGKLEESPDNDGF
LKAPCRMKVSIPTKALELMDMQTFKAEPPEKPSAFEPAIEMQKSVPNKALELKNEQTLRADQMFPSESKQKKVEENS
WDSESLRETVSQKDVCVPKATHQKEMDKISGKLEDSTSLSKILDTVHSCERARELQKDHCEQRTGKMEQMKKKFCVL
KKKLSEAKEIKSQLENQKVKWEQELCSVRLTLNQEEEKRRNADILNEKIREELGRIEEQHRKELEVKQQLEQALRIQDIEL
KSVESNLNQVSHTHENENYLLHENCMLKKEIAMLKLEIATLKHQYQEKENKYFEDIKILKEKNAELQMTLKLKEESLTKR
ASQYSGQLKVLIAENTMLTSKLKEKQDKEILEAEIESHHPRLASAVQDHDQIVTSRKSQEPAFHIAGDACLQRKMNVD
VSSTIYNNEVLHQPLSEAQRKSKSLKINLNYAGDALRENTLVSEHAQRDQRETQCQMKEAEHMYQNEQDNVNKHTE
QQESLDQKLFQLQSKNMWLQQQLVHAHKKADNKSKITIDIHFLERKMQHHLLKEKNEEIFNYNNHLKNRIYQYEKEK
AETENS

5.2 Predicted T-cell Epitopes

5.2.1 Class I MHC restricted T-cell epitopes

BIMAS tool was used to find out the class I MHC binding peptides. BIMAS predict T cell epitope based on $T_{(1/2)}$ which estimates the half time of dissociation of a molecule. We have considered different $T_{(1/2)}$ values to evaluate the T cell epitopes. Number of epitopes predicted at different $T_{(1/2)}$ values were shown in Table 4 and Fig 1. We found that the number of predicted epitopes is decreasing with increase in $T_{(1/2)}$ values. Considering $T_{(1/2)}$ value equal to 100 and 200 is more stringent as less number of epitopes were predited while at 1, 5, 10 and 20, a large number of epitopes were predicted. Predicted epitopes were comparative in case of $T_{(1/2)}$ at 50 and 100. In other study done in our lab on influenza virus, $T_{(1/2)}$ at 50 is found to be optimal for

prediction of epitopes. Finally we have considered the epitopes which were predicted at $T_{(1/2)}$ value equal to 50. These epitopes were used to generate the overlapping peptide fragments. Finally we have found 20 peptides containing two or more MHC class I restricted T-cell epitopes which can be considered further for evaluation of immunogenic response (TABLE 5).

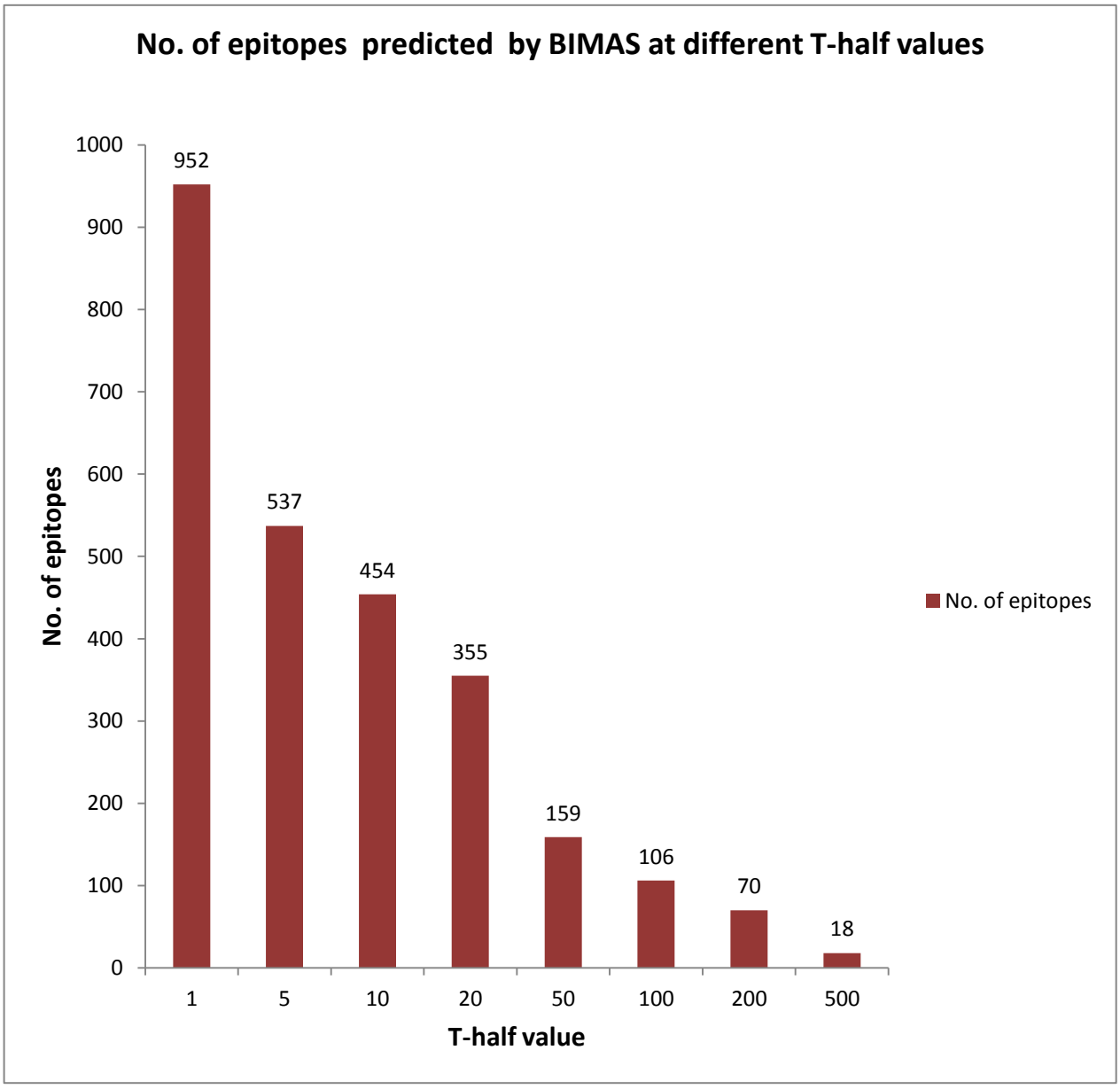


Fig 1: MHC Class I restricted T-cell epitope prediction by BIMAS at different T-half values

TABLE 4: Predicted T-cell epitopes for Class I MHC.

Predicted T-Half values	T=1	T=5	T=10	T=20	T=50	T=100	T=200	T=500
Total no of epitopes Predicted	952	537	454	355	159	106	70	18
No of unique epitopes	382	0	0	0	0	0	0	0
No of epitopes common to all T-half values	11	11	11	11	11	11	11	11

TABLE 5: Immunogenic peptides containing overlapping MHC Class I T-cell Epitopes

Sr. No.	IMMUNOGENIC PEPTIDE	NO. OF OVERLAPPING EPITOPES
1.	GHEEVVTFLVDRKCQL	4
2.	HQEACANILIDSGADINL	4
3.	SEQIVEFLLIKNA	2
4.	CVARVTSNKTKVLEKGRSKMI	4
5.	SRSLFESSAKIQVCIPESIYQKVMEINREVEEPPKK	7
6.	SLCETVSQKDVCLPK	2
7.	IPTKALELMDMQTF	2
8.	KPSAFEPAIEM	2
9.	CRMKVSIPTKALELMDMQTF	4
10.	SLRETVSQKDVCVPK	2
11.	SLSKILDTVHSCERAR	2
12.	LQKDHCEQRTGKMEQMKKKFCVLKKKLSEAKEIKSQLENQKVK	6
13.	LTLNQEEEKRRNADILNEKI	3
14.	IEEQHRKELEVKQQLEQALRIQDIELK	5
15.	THENENYLLHENCML	2
16.	KYFEDIKILKEKNAELQMTLKLKEESLTKRASQYSGQLKVL	6
17.	HQPLSEAQRKSKSLKINLNYAGDALR	4
18.	QRDQRETQCQMK	2
19.	QQESLDQKLFQLQSKNMWLQQQLVHAHKK	8
20.	LERKMQHHLLKEKNEEI	3

5.2.2 Class II MHC restricted T-cell epitopes

NetMHCII 2.2 server was used to predict binding of peptides to HLA-DR, HLA-DQ, HLA-DP MHC class II alleles. The epitopes were predicted according to their binding strength to different HLA alleles and were categorized into strong binders and weak Binders. Strong Binders which bind to more than three HLA alleles were only selected. The predicted epitopes are shown in Table 6.

Six immunogenic peptides containing overlapping epitopes were obtained and are shown in Table 7. The length of immunogenic peptide varied from 11-18 amino acids residues.

TABLE 6: MHC Class II Restricted T-cell Epitopes predicted by NetMHCII

MHC Class II Restricted T-cell Epitopes (NetMHCII)	
EPITOPE	**No. of HLA Alleles**
AVYSEILSV	**3**
FESSAKIQV	**4**
FHHIHEQIM	**6**
FCVLKKKLS	**3**
FLVDRKCQL	**5**
FQLQSKNMW	**8**
FLLIKNANA	**6**
IAMLKLEIA	**7**
IKNANANAV	**7**
ILIDSGADI	**6**
KLLSHGAVI	**2**
LFQLQSKNM	**5**
LIAENTMLT	**5**
LKATCGMKV	**4**
LKLEIATLK	**4**
LKVLIAENT	**3**
LHYAVYSEI	**3**
SLFESSAKI	**4**
YFEDIKILK	**4**
WLQQQLVHA	**3**

TABLE 7: Immunogenic peptides containing overlapping MHC Class II T-cell epitopes

Sr. No.	IMMUNOGENIC PEPTIDE	NO. OF EPITOPES
1.	LHYAVYSEILSV	2
2.	FLLIKNANANAV	2
3.	SLFESSAKIQV	2
4.	LFQLQSKNMWLQQQLVHA	3
5.	LKVLIAENTMLT	2
6.	AMLKLEIATLK	2

5.2.3 Common immunogenic peptide selection for both Classes of MHC molecule

Common immunogenic peptides were found by identifying and selecting the common region of both MHC Class I and MHC II immunogenic peptides.

These immunogenic peptides were 11 to 18 amino acid long. These immunogenic peptides are shown in Table 8.

TABLE 8: MHC Class I and MHC Class II immunogenic peptides.

Sr. No.	MHC CLASS I IMMUNOGENIC PEPTIDES	MHC CLASS II PEPTIDES
1.	SRSLFESSAKIQVCIPESIYQKVMEINREVEEPPKK	SLFESSAKIQV
2.	QQESLDQKLFQLQSKNMWLQQQLVHAHKK	LFQLQSKNMWLQQQLVHA

5.3. Predicted B-cell epitopes

Kolaskar & Tongaonkar Antigenicity and Bepipred Linear epitope prediction algorithms were used for prediction of B-cell epitopes. B cell epitopes which were predicted by these two methods are given in (Table 9 & Table 10). We have selected those peptides which contained epitopes predicted by both methods. Finally, eleven immunogenic peptides were found and their length varied from 7 to 18 amino acid residues (Table 11). We have also found one peptide which contains MHC Class I restricted T-cell epitopes and a B-cell epitope (Table 12).

TABLE 9: B-cell epitopes predicted by Kolaskar & Tongaonkar Antigenicity Method

Kolaskar & Tongaonkar Antigenicity
EPITOPE
RTALHWACVNGHEEVVTFLVDRKCQLDVLDG
MKALQCHQEACANILIDS
INLVDVYGNTALHYAVYSEILSVVAKLLSHGAVIEVHNKASLTPLLLSIT
EQIVEFLLINK
VNKYKCTALMLAVCHGSSEIVGMLLQQNVDVFAADICGVTAEHYAVTCGFHHIHE
AESLVEK
AAPLVER
AESLVEK
AASLVEG
KIQCLEK
KTGCVARV
TKVLEK
MIACPTK
EYSCDSR
LFESSAKIQVCIPESIYQKV
KPSAFKP
ESLCETV
ESLCETV
QKDVCLPKAT
GMKVSIPKALEL
KSVPNKLE
DEILPSE
QKDVCLPKAA
VKDGLLKN
GMKVSIPTKALE
QKSVPNKALE
DEILPSE
ESLCETV
QKDVCLPKAT
LKAPCRMKVSIPTKALE
QKSVPNKALE
QKDVCVPKAT
LSKILDTVHSCE
KDHCEQ
KKFCVLKKK
EQELCSVRLT
ELEVKQQLEQALRIQDIELKSVESNLNQVSHT
YLLHENC
KKEIAMLKLEIATLKHQY
SQYSGQLKVLIA
ESHHPRLASAVQDHDQIVTSR
EPAFHIAGDACLQRK
NVDVSST
NNEVLHQPLSE
SLKINLN
NTLVSEH
SLDQKLFQLQ
QQQLVHAHK
TIDIHFL
QHHLLK

TABLE 10: B-cell Epitopes predicted by Bepipred Linear Epitope Prediction method

Bepipred Linear epitope prediction
EPITOPE
KNHQNTNPEGTSAGTPDEAAPLAERTPDTAESLVEKTPDEAAPLVERTPDTAESLVEKTPDEAASLVEGTSD
ATSGKFEQSAEETPREITSPAKETSEKFTWPAKGRPRKIAWEKKEDTPREIMSPARKIAWEKETSEKFTWAAKGRPKKETPVKT
PTKESSTKASANDQRFPSESKQEEDEEYSC
REVEEPPKKPSAFK
EMQNSVPN
TLRADPMFPPESKQKDYEENSWDSE
ATHQKEIDK
KLEESPNNKDGL
TFKAEPPGKPSAFEPATEMQKSVPN
ILPSESKQKDYEENSWDTE
ATHQKEIDK
GKLEGSPVKD
FKAEPPEKPSAFEPA
EMQKSVPN
ILPSESKQKDYEESSWDSE
ATHQKEIDK
GKLEESPDNDHF
FKAEPPEKPSAFEPA
EMQKSVPN
MFPSESKQKKVEENSWDSESLRETVSQ
VCVPKATHQKEMDK
GKLEDSTS
KDHCEQRTGKME
AKEIKSQLEN
QEEEKRR
QYQEKENKY
LTKRASQYS
KEKQDKEI
QIVTSRKSRKSQEPAFH
LSEAQRKS
HAQRDQRETQCQM
HMYQNEQDNVNKHTEQQESL
KKADNKSK
YEKEKAETENS

TABLE 11: The common immunogenic peptides containing B-cell epitopes predicted by two methods (Kolaskar & Tongaonkar and Bepipred)

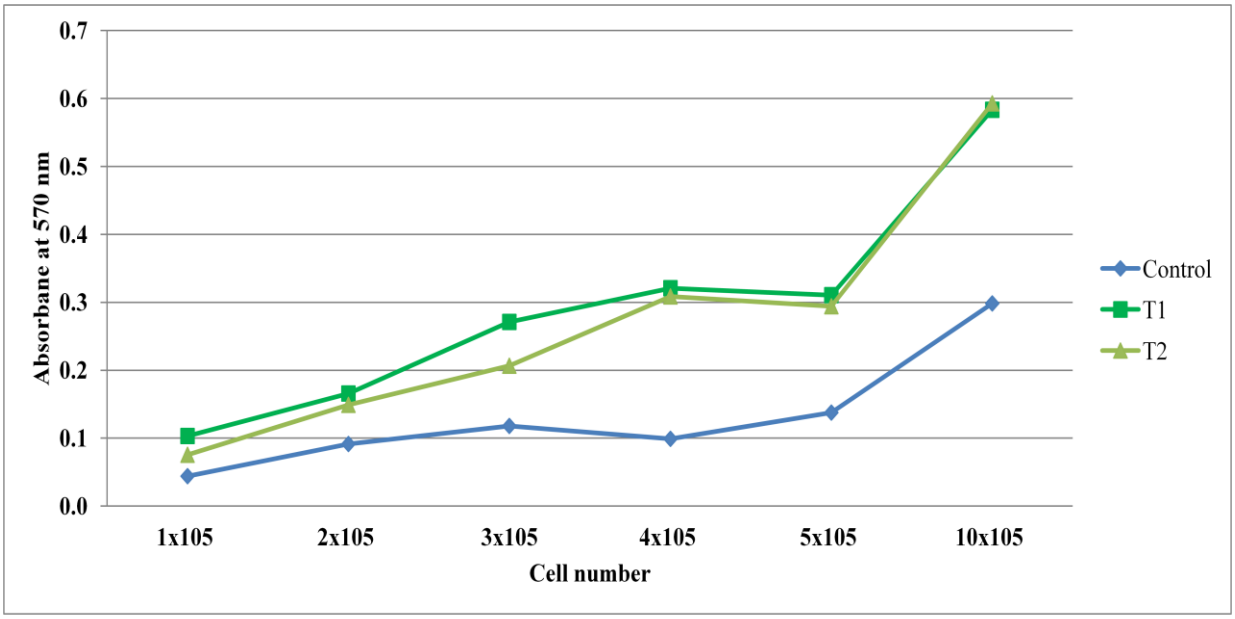
Sr. No.	Common Immunogenic Peptides containing B-cell epitopes
1	AESLVEKTPDEAAPLVER
2	AESLVEKTPDEAASLVEG
3	KPSAFKP
4	REVEEPPKKPSAFK
5	DEILPSE
6	EMQKSVPN
7	QKSVPNKALE
8	EMQKSVPN
9	QKDVCVPKAT
10	KDHCEQRTGKME
11	QIVTSRKSRKSQEPAFH

TABLE 12 : Common immunogenic peptide containing MHC Class I restricted T-cell epitopes and B-cell epitope

Sr. No.	IMMUNOGENIC PEPTIDE CONTAINING MHC CLASS I RESTRICTED T-CELL EPITOPES	IMMUNOGENIC PEPTIDE CONTAINING B-CELL EPITOPES
1	LQKDHCEQRTGKMEQMKKKFCVLKKKLSE AKEIKSQLENQKVK	KDHCEQRTGKME

5.4 Optimization of protocol for PBMC Proliferation Assay at preliminary steps (MTT Assay)

In order to optimize the effect of mitogen, we have done the lymphocyte proliferation assay. We have taken different cell numbers at two different concentration (5µg/ml and10µg/ml) of



(T1= 5µg/ml , T2=10ug/ml)

concanavalin-A(ConA) mitogen (Fig 2). We have found that with increase in cell number, there is more proliferation as compared to control. We did not observe significant difference in two different concentration of mitogen.

Fig 2: Proliferation assay of peripheral blood mononuclear cells using MTT assay

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CONCLUSION

Immunoinformatics approach plays an important role in peptide based vaccine against infectious disease and cancer. In the present study we have identified consensus sequence of NY-BR-1 breast cancer antigen for prediction of peptide containing T-cell and B-cell epitopes. Based on prediction algorithm, we identified twenty and six immunogenic peptides containing epitopes for MHC Class I and II respectively which ranges from 11 to 43 amino acid residues. Finally, we found two peptides which are commonly present in both class I and II MHC. We have also conducted study to predict the B cell epitope using immunoinformatics tool. Eleven immunogenic peptides containing B cell epitopes were finally selected ranging in length from 7 to 18 amino acid residues. We also identified one immunogenic peptide which contains Class I MHC restricted T-cell epitopes and a B-cell Epitope.

These immunogenic peptides containing T and B cell epitopes can be used for further study to assess the affinity of immunogenic peptide to MHC molecule by structural analysis and molecular modelling. Further, study can be carried out to assess the potential of these peptides for immunogenic response in the PBMC by T-cell proliferation assay (MTT assay) and cytokine production assay.

Hence, these immunogenic peptides may be considered as interesting candidates in designing breast cancer vaccine.

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SUMMARY

The past decades have seen advances in the diagnosis and treatment of breast cancer. Despite this progress, breast cancer is still a leading cause of cancer-related deaths among women. Although tumorectomy, radiotherapy, chemotherapy and hormone replacement therapy have been used for the treatment of breast cancer, there is no effective therapy for patients with invasive and metastatic breast cancer. The characterization of tumor antigens recognized by immune effector cells has opened the perspective of developing therapeutic vaccines in the field of breast cancer. Development and approval of new vaccines are the hope for cure of different cancers. Identification of effective epitopes in these tumor antigens will significantly rationalize the development of epitope-based vaccines.

NY-BR-1 is classified as a diferentiation antigen of the mammary gland. A recent immunohistochemical study with a limited number of breast carcinoma lesions has shown that NY-BR-1 is expressed in about 60% of breast carcinoma and has confirmed the breast epithelium specificity (Varga *et al*., 2006). Due to this organ-specificity, NY-BR-1 represents an attractive target for cancer immunotherapy and can be potentially used for diagnostic purposes in surgical pathology. NY-BR-1 breast cancer antigen meets most of the criteria required to be utilized as a target for T-cell-based immunotherapy.

Present study is focused on the prediction of potential T and B-cell epitopes of NY-BR-1 breast cancer antigen to identify immunogenic peptides which can act as target for vaccine design against breast cancer. Using various immunoinformatic tools, we were able to identify long stretch of immunogenic peptides which cover large number of B-cell and T-cell epitopes. BIMAS and NetMHCII were used for prediction of Class I and II MHC restricted T cell epitopes respectively. Twenty and six immunogenic peptides containing epitopes were identified for MHC Class I and II respectively. Finally we found two peptides which are commonly present in both class I and II MHC. We have also conducted study for B-cell epitope prediction using immunoinformatics tool. Kolaskar & Tongaonkar Antigenicity and Bepipred Linear epitope prediction algorithms were used for prediction of B-cell epitopes. Eleven immunogenic peptides containing B cell epitopes were finally selected. We also identified one immunogenic peptide which contains MHC Class I restricted T-cell epitopes and a B-cell Epitope.

We have found different immunogenic peptides containing T and B cell epitopes based on immuniformatics approach. Their immunogenic activity may be assessed further for the affinity of immunogenic peptide to MHC molecule by structural analysis and molecular modelling.

These peptides can be selected for chemical synthesis and then evaluated for immunogenic response in the PBMC by T-cell proliferation assay (MTT assay) and cytokine production assay. Based on these studies, the immunogenic peptides may be considered as interesting candidates in designing breast cancer vaccine.

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