EXPRESSION OF MONOCLONAL ANTIBODY IBMR3 ANTIGEN IN BREAST CANCER TISSUE

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EXPRESSION OF MONOCLONAL ANTIBODY IBMR3 ANTIGEN IN BREAST CANCER TISSUE

by

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LIST OF ABBREVIATIONS

2D-PAGE	Two-Dimensional Polyacrylamide Gel Electrophoresis
ADCC	Antibody-Dependent Cellular Cytotoxicity
AJCC	American Joint Committee on Cancer
AML	Acute Myelogenous leukaemia
BSA	Bovine Serum Albumin
CID	Collision Induced Dissociation
DAB	3,3-Diaminobenzidine
DMSO	Dimethyl Sulfoxide
DTT	Direct Tissue Trypsinisation
EB	Extraction buffer
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor
EU	European Union
FASP	Filtered Aided Sample Preparation
FBS	Fetal Serum Albumin
FCS	Fetal calf serum
FFPE	Formalin-Fixed and Paraffin Embedded
FITC	Fluorescein Isothiocyanate
GC	Granular Component
H&E	Hematoxylin and Eosin
HER2	Human Epidermal Growth Factor Receptor 2
IgM	Immunoglobin M
HPF	High Power Magnification
HRP	Horseradish Peroxidase
IARC	International Agency for Research on Cancer
IBMR3	Ishak Bin Mat and Mary Ritter 3
ICC	Immunocytocemistry

IF	Immunofluorescence
IHC	Immunohistochemistry
MAb	Monoclonal Antibody
MS	Mass Spectrometry
MW	Molecular Weight
NGS	Nottingham Grading System
NK	Natural Killer
NRS	Normal Rabbit Serum
PBS	Phosphate Buffered Saline
PBMC	Peripheral Blood Mononuclear Cells
PR	Progesterone Receptor
PTM	Post-Translational Modification
PVDF	Polyvinylidene Difluoride
RT	Room Tempreture
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SDS–PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SPSS	Statistics Package for Social Science
TA	Tumour Antigen
TBS	Tri-Buffer Saline
TBST	Tri-Buffer Saline Tween 20
TCA	Trichloroacetic Acid
TFA	trifluoroacetic acid
TMA	Tissue Microarray
TNBC	Triple Negative Breast Cancer
WHO	World Health Organisation
VSMC	Vascular Smooth Muscle Cell

PENGEKSPRESAN ANTIGEN ANTIBODI MONOKLON IBMR3 DALAM TISU KANSER PAYUDARA

ABSTRAK

Pengesanan beberapa jenis kanser boleh dilakukan dengan menggunakan antibodi monoklon (AbM). Kajian menunjukkan bahawa jenis-jenis kanser yang berbeza mempunyai protein tak normal yang tertentu yang boleh dikenal pasti oleh AbM. IBMR3 AbM dibangunkan daripada peptida sintetik yang berhubung dengan jujukan asid amino terpilih daripada reseptor interleukin-4 manusia. Kajian ini adalah untuk mengesan ekspresi IBMR3 antigen dalam karsinoma termasuk Manusia carcinoma berhubung dgn pangkal tekak epitelium (HEp-2), adenokarsinoma kolon Manusia (HT-29) dan tisu kanser payudara. Ungkapan IBMR3 antigen dikesan pada HEp-2 dan HT-29 menggunakan ujian berbentuk tidak langsung pewarnaan, aliran sitometri pewarnaan. Hasil kajian menunjukkan persatuan kemungkinan IBMR3 antigen ungkapan dalam karsinoma (kanser sel-sel epitelium) dengan pewarnaan cytoplasmic positif. HEp-2 dan HT-29 telah dipisahkan melalui 1-D immunoblot dan keputusan menunjukkan dua band dengan berat molekul (MWS) 45 dan 25 kDa dalam HT-29 dan satu band dengan MWS 25 kDa dalam HEp-2. IBMR3 antigen ungkapan itu dikesan pada 48 sampel kanser payudara Malaysia dan microarray (TMA) slaid tisu kanser payudara 430 dan 132 tisu payudara yang normal bersebelahan dengan kanser payudara melalui IHC. Pengekspresan antigen ini amat ketara berkait dengan gred histologi, peringkat tumor, dan metastasis nodus limfa. Pengekspresan IBMR3 yang tinggi diperhatikan pada tisu kanser payudara gred I

berbanding pengekspresan yang diperhatikan pada gred III pada kedua-dua sampel kanser payudara pesakit Malaysia ($P < 0.003$) dan slaid TMA ($P < 0.01$). Dapatan daripada kajian ini menunjukkan hubungan yang signifikan antara pengekspresan antigen IBMR3 dan ciri-ciri klinikopatologi bagi. Tisu karsinoma payudara manusia berjaya diasingkan melalui imunoblot 1-D. Keputusan menunjukkan dua jalur dengan berat molekul (BM) 45 dan 25 kDa. Jalur reaktif yang dipotong daripada elektroforesis gel dianalisis menggunakan LC-MS/MS. Keputusan daripada kajian ini mencadangkan bahawa antigen IBMR3 mungkin terdiri daripada protein zeta 14-3-3 yang disahkan melalui fungsi biologinya di dalam kanser payudara dan laluan interaksinya dengan IL-4R. Penemuan ini menggambarkan hubungan yang signifikan di antara pengekspresan antigen IBMR3 dan jenis-jenis kanser epitelium yang lain. Antigen IBMR3 berkemungkinan memainkan peranan dalam perkembangan tumor dan pengekspresannya berkemungkinan mempunyai nilai yang signifikan dalam prognosis kanser.

EXPRESSION OF MONOCLONAL ANTIBODY IBMR3 ANTIGEN IN BREAST CANCER TISSUE

ABSTRACT

The detection of several types of cancer is possible by using monoclonal antibody (MAb). Studies have shown that different types of cancer have related specific abnormal proteins, which are identified by MAb. IBMR3 is a MAb which was raised against synthetic peptides that are associated with the selected amino acid sequence of human interleukin-4 receptors. This research is to detect the expression of IBMR3 antigen in carcinoma including Human laryngeal epithelial carcinoma (HEp-2), Human colon adenocarcinoma (HT-29) and breast cancer tissues. The expression of IBMR3 antigen was detected on HEp-2 and HT-29 using indirect immunofluorescent staining, flow cytometry staining. The results showed the possible association of IBMR3 antigen expression in carcinoma (epithelial cancer cells) with positive cytoplasmic staining. HEp-2 and HT-29 were separated via 1-D immunoblot and the results showed 2 bands with molecular weights (MWs) of 45 and 25 kDa in HT-29 and one band with MWs of 25 kDa in HEp-2. IBMR3 antigen expression was detected on 48 samples of Malaysian breast cancer and microarray (TMA) slides of 430 breast cancer tissues and 132 normal breast tissues adjacent to breast cancer through IHC. The expression of this antigen significantly correlated to the histological grades, tumour stages, and lymph node metastases. High IBMR3 expression was observed in grade I cancerous breast tissues compared with the

expression observed grade III in both Malaysian breast cancer samples (P < 0.003) and TMA slides (P < 0.01). Human breast carcinoma tissues were separated via 1-D immunoblot. Results showed 2 bands with molecular weights (MWs) of 45 and 25 kDa. The reactive bands that were excised from gel electrophoresis were subjected to liquid chromatography-tandem mass spectrometry. IBMR3 antigen was identified using 1-DE gel coupled with LC-MS/MS successfully. The results of this study suggested that IBMR3 antigen could be 14-3-3 zeta/delta protein, which was supported by its biological function in breast cancer and it has interaction pathway with IL-4R. The findings from this study exhibited a significant relationship between IBMR3 antigen expression and different types of epithelial cancer cells. IBMR3 antigen may play a role in tumour development and that its expression may have a significant value in cancer prognosis.

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Cancer is a disease of a cell or a group of cells that divides and replicates uncontrollably because of the accumulation of both epigenetic and/or genetic changes (Verma & Singh, 2013). Cancer types depend on the origin site of the malignant cells, the histology, or cell analysis (grading), and the extent of the disease (staging). Cancers are classified into 3 main groups, namely, carcinomas, sarcomas, and leukemias or lymphomas. Cancers that developed in epithelial tissues are called carcinomas, which are detected in approximately 90 % of human cancers. Cancers that developed in connective tissues are called sarcomas, which are rare in humans. Leukemias and lymphomas are cancers that developed in cells that normally mature in the bloodstream and in the lymphatic system respectively (Cooper, & Hausman, 2000).

Most carcinomas affect secretory organs or glands, such as breasts that produce milk, the lungs that secrete mucus, colon, prostate, and bladder (Hayat, 2004). Breast cancer tissues are rich in epithelial cells (Rezaul et al., 2010). The high morbidity of breast cancer is related to late diagnosis in which cancer has reached aggressive stages (Jassem et al., 2013). It is a common cause of cancer death among women (Chahil et al., 2015; Harhra & Basaleem, 2012), followed by colon cancer (Hamid et al., 2012; Zainal Ariffin & Nor Saleha, 2011). Several colon cancer cell lines originate from the epithelial region, including the HT-29 cells (Aggarwal et al., 2015). The HT-29 human colon carcinoma cell lines are widely used to model the

physiological and immune function of intestinal epithelial cells (Bruno & Kaetzel, 2005). Meanwhile, the HT-29 human colon adenocarcinoma cell lines offer favourable experimental systems to investigate the factors involved in epithelial cell differentiation (Cohen, Ophir, & Shaul, 1999). HT-29 cells correspond to well-differentiated colorectal adenocarcinoma, which is grade one (I) colorectal adenocarcinoma (Rimkus et al., 2006). HT-29 human colon carcinoma cell lines are widely used to model the physiological and immune functions of intestinal epithelial cells (Bruno & Kaetzel, 2005), Likewise, HEp-2 is a human laryngeal epithelial carcinoma cell line, which is derived from laryngeal carcinoma cells and is often utilised as a model in carcinogenicity and mutagenesis tests (Lima et al., 2005). HEp-2 cells are related to poor differentiation, advanced clinical stages, and tumour grade (Tian et al., 2014).

Cytokines and their receptors are important contributors to tumour development. Interleukin 4 (IL-4) is a pleiotropic cytokine that demonstrates opposite effects on tumour growth including the increase and decrease of the proliferation and survival of malignant cells (Li et al., 2009). Interleukin-4 receptor α (IL-4Rα) is expressed in numerous cancer cell lines derived from non-hematologic human malignancies, such as melanoma, pancreatic adenocarcinoma, glioblastoma, colon carcinoma, gastric carcinoma, breast carcinoma, ovarian carcinoma lung carcinoma, head and neck cancers, and renal cell carcinoma. IL-4R is expressed in many human epithelial cancer cells; however, the binding characteristics, structure, function, and signal transduction through the IL-4R in cancer cells are not known. Human breast carcinoma cell lines express high-affinity IL-4R. A previous study demonstrated that the increase in IL-4R expression renders HT-29 and HEp-2 cells to

be more sensitive to IL-4 and that low concentrations of IL-4 are required to exert its effect on cell proliferation (Suriza, Musa, & Mat, 2006).

To date, various targeted therapies are evaluated in cancer patients. Monoclonal antibody (MAb) is one of the most promising approaches to enhance patient survival outcome (Bellati et al., 2011). MAb is prepared from a single clone (hybridoma) of white blood cells, as described by Köhler and Milstein (1975). The use of MAb in cancer treatment is focused on targeting tumour cells that express tumour-associated antigen (Oldham & Dillman, 2008). MAb is also used to antagonize receptor signaling pathways, which are essential in tumour cell migration, survival, and proliferation. MAb mainly blocks key receptors on tumour cell surfaces. It is also used to recruit the immune system cellular arm on the transformed cell. Although MAb is synthetically produced, it mimics naturally produced antibodies as part of the immune system's response to disease (Kewal et al., 2014).

The IBMR3 is a MAb with an IgM isotype. It is produced from 4 peptides of interleukin 4 receptor. IBMR3 antibody showed weak positive result with resting PBMC, but expression upregulation was observed in activated PBMC using anti-CD3 and IL-4 (Mat, 1992). The IBMR3 antigen expression was analysed using flow cytometry on normal peripheral blood and leukaemic cells. The results showed no staining on the surface of normal resting PBMC, but high expression was observed at the cytoplasm of the cell. In the transformed haemopoietic cells, 9 acute myelogenous leukaemia (AML), 2 acute lymphoid leukaemia (ALL-T) and 6 ALL-B cells showed intense cytoplasmic staining and negative staining on the surface. The 2 AML cells showed staining in both cytoplasmic and surface regions (Hara & Mat, 2004). Previous study showed that IBMR3 antigen was expressed significantly

higher at the cytoplasm of lymphocytes and monocytes when compared with their surface. The expression of IBMR3 antigen was differentially expressed in malignant haemopoietic cells (acute leukaemia) compared to normal PBMC (Hara, 2002).

According to Adams and Weiner (2005), antibodies are used for cancer therapy by directing them to cell antigens on the cell surface that is associated with tumour stroma, as well as to antigens on the tumour-associated vasculature and ligands, which support tumour growth in both circulating malignant cells and solid tumours. Tumour antigen, which is targeted by MAb, is one of the most successful new therapies (Lee et al., 2011). Previous studies indicated that MAb is important in the biomedical research of targeted drug delivery systems, particularly in the treatment of cancer, as well as metabolic and hormonal disorders (Deb et al., 2013). Many antibodies were approved by the Food and Drug Administration (FDA) of United States for cancer treatment. FDA approved palbociclib (Ibrance) in February 2015. Palbociclib is a kinase inhibitor that is used in combination with letrozole for treatment of postmenopausal women with estrogen receptor-positive and human epidermal growth factor receptor 2 (HER2)-negative advanced breast cancer as initial endocrine-based therapy for metastatic disease (Beaver et al., 2015). Therapeutic predictions are now identifiable because of the diagnosis from proteome analysis (Donadio et al., 2011). Studies of breast cancer-derived proteins that use electrophoretic separation and immunoblotting techniques have been published and widely available (Osborne & Brooks, 2006). O’Farrell developed a technique for protein separation (O’Farrell, 1975).

The basic process of protein separation consists of identifying protein isoelectric point and then analysing protein according to its size. This process is conducted by two-dimensional electrophoresis (2DE) (Kondo, 2010) and paves the way for a broader study of more than 1000 protein samples in a single experiment from a cell or tissue extract. In previous studies, SDS-PAGE and immunoblot analysis were used to determine and represent the shared agglutinin-binding glycoproteins in clinical tumour samples and breast cell lines (Osborne & Brooks, 2006). Many of these studies detect different protein levels in healthy and diseased tissues. The expression profiling of tissue protein is potent in discovering new biomarkers to improve breast cancer prognosis, diagnosis, and staging (Freitas et al., 2013).

1.2 PROBLEM STATEMENT

IBMR3 MAb was raised against synthetic peptides that similar to certain amino acid sequences in human IL-4R (Mat, 1992). Cytokines and their receptors are vital factors for tumour advancement. IL-4R is expressed in many human epithelial tumours. IL-6, IL-4 and IL-8 levels are increased among patients with prostate, breast or colon cancer. The significance of the IL-4R overexpression in epithelial cancer cells has not been elucidated.

The significance of IL-4R overexpression in epithelial cancer cells is not elucidated as well as the binding function and correlation of IBMR3 expression with clinical stage and grade in cancer cells remain unclear. Previous study found that there was strong expression of IBMR3 antigen in early stage of breast cancer development and the expression ceased once the tumour metastasised to lymph node

(Hara, 2002). Unfortunately, only three samples were previously analysed which considered too little. Therefore, in order to detect IBMR3 antigen expression on larger sample size of breast cancer, further study is demanded. The preliminary experiment was performed on poorly and well differentiated cell lines, HEp-2 and HT-29 cells respectively. Moreover, IBMR3 antigen expression was more prominent in malignant haemopoietic cells (acute leukaemia) as compared with normal PBMC (Hara, 2002). The epithelial cancer cell lines, HEp-2 and HT-29 were selected to compare the expression of IBMR3 antigen between haemopoietic cell and carcinoma. There is also no study available on the IBMR3 antigen and its role on cancer. Hence, this study aims to provide better understanding on the function of this protein its potential implication on cancer.

1.3 OBJECTIVES OF THE STUDY

1.3.1 General Objective

This study aims to investigate the expression of IBMR3 antigen in colorectal cancer cell line, laryngeal cancer cell line and breast cancer tissues and to profile the IBMR3 antigen in breast cancer tissue.

1.3.2 Specific Objectives

1. To detect the expression of IBMR3 antigen in epithelial cancer cell lines (HEp-2 and HT-29).

2. To recognise IBMR3 antigen expression in breast cancer tissues of Malaysian patients.

3. To determine the association between the IBMR3 antigen expression level with patient clinicopathological features.

4. To separate and detect IBMR3 antigen in breast cancer tissues using immunoblot.

5. To identify the candidates for IBMR3 antigen in breast cancer tissues using liquid chromatography- tandem mass spectrometry.

1.4 HYPOTHESIS OF THE STUDY

1. The expression of IBMR3 antigen is positive in colorectal cancer cell line, laryngeal cancer cell line and breast cancer tissues.
2. The expression of IBMR3 antigen is associated with cancer development.
3. The IBMR3 antigen has intracellular connections with IL-4 receptors.

CHAPTER 2

LITERATURE REVIEW

2.1 MONOCLONAL ANTIBODIES

MAbs are the protein molecules which were synthetically created from hybridoma cells (stable cell lines) by fusing antibody-producing cells from the immunised animals with cells that confer immortality and yield high antibody production (Köhler & Milstein, 1975) or by recombinant deoxyribonucleic acid (DNA) technology (Zola, Thomas, & Lopez, 2013). MAb technology is an important development in creating specific serologic reagents within a large diversity of antigens to produce several types of highly specific and reproducible immunological assays for fast and accurate diagnosis (Deb et al., 2013). Previous study illustrated that the hybridoma cell culture which yields the monoclonal antibodies shows the possibility of an unrestrained supply of reagent (Nelson et al., 2000).

2.1.1 Monoclonal Antibody Production

MAbs were produced by engineering hybridoma cells to form the desired antibody in a large amount (Pandey, 2010). Köhler and Milstein (1975) discovered the way to create monoclonal antibodies from hybridoma by fusing malignant myeloma cells with antibody-producing B-cells (Figure 2.1). The first stage in Köhler and Milstein's technique for producing the monoclonal antibodies involves immunisation of an experimental animal with the antigen of interest. In other words, the mouse is injected with an antigen. As a result, the mouse initiates an immune response and produces antibodies specific to the antigen. Spleen cells from the immunised mouse are taken and hybridized with myeloma cells.

Hybridomas which resulted from the hybridization have the survival chance. Generally, the spleen lymphocyte has a limited life span. Therefore, any B-cells which do not merge with myeloma will die in the culture. The isolated hybridoma cells are examined for their specificity to the antigen. This is because each hybridoma originated from B-cell creates copies for only one antibody (Köhler & Milstein, 1975).

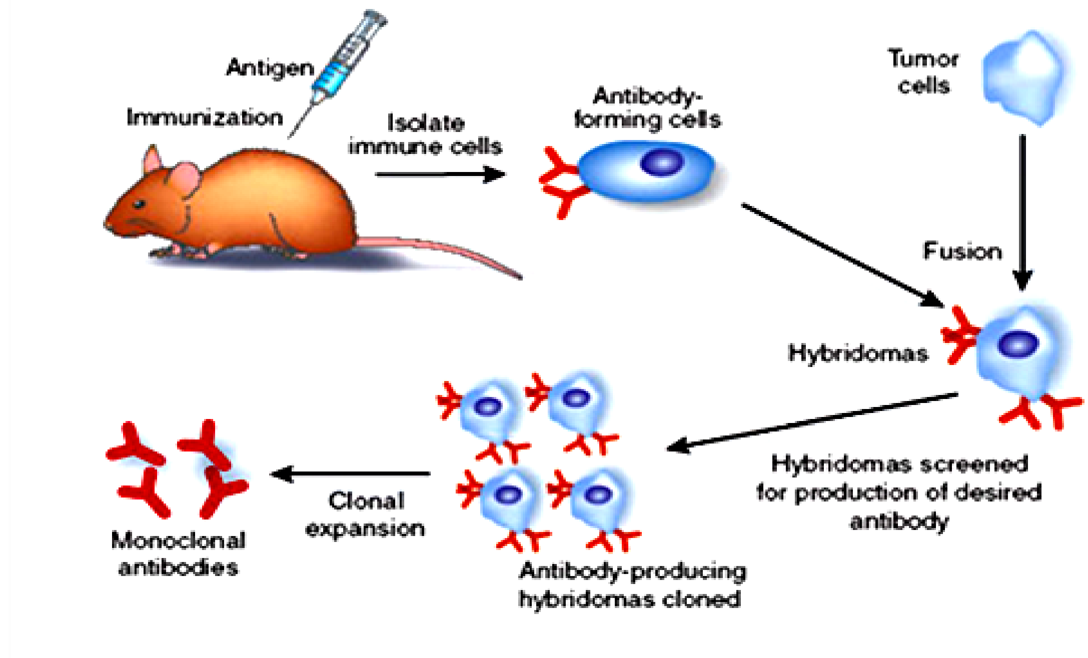

Figure 2.1: The production of monoclonal antibodies from a mouse spleen cell. Adapted from Michnick and Sidhu (2008).

2.1.2 Application of Monoclonal Antibodies

MAbs are widely used in biomedical science to identify proteins, carbohydrates and nucleic acids, as well as to figure out molecules which control cell replication and differentiation. The application of monoclonal antibodies includes the following sections.

2.1.2(a) Diagnostic Application of Monoclonal Antibody

MAbs can be used in a clinical laboratory, diagnostic tests such as various immunoassays, radioimmunoassays, particle agglutination, immunofluorescent antibody assays, enzyme-linked immunosorbent assays and immunohistology (Payne et al., 1988). MAbs are applied in diagnostic histopathology and these molecules are used for the purpose of classifying tissues and tumours based on their expression in defined markers, thus reflecting tissue or cellular genesis. For instance, monoclonal antibodies of specific organ-associated antigens such as the prostate specific antigen, human chorionic gonadotrophin, fetoprotein, placental alkaline phosphatase and others are capable of assisting the pathologist to establish the nature of a primary tumour (Nelson et al., 2000).

The histopathological staining using haematoxylin and eosin is not sufficient for detecting a small number of invasive or metastatic cells (Winnard et al., 2008). HER-2/neu protein is known as a family member that belongs to growth factor receptors. Previous research showed that identification of the oncogene overexpression, HER-2/neu (c-erbB-2) in breast cancer lead to develop an immunotherapy approach to dealing with this disease based on using the MAb (Hermiston & Kirn 2005)

2.1.2(b) Prognostic Application of Monoclonal Antibody

There are certain markers which are detected by MAbs provide useful information on prognosis in cancer patients. For instance, detecting the anti-apoptosis protein BC-2 has been described to be an indicator of poor prognostic with a diversity of tumour kinds such as prostate and ovarian cancers, as well as for Hodgkin's s and non-Hodgkin's lymphomas. Contrarily, downregulating of the cyclin-dependent kinase inhibitor is associated with poor prognosis in non-Hodgkin's lymphomas, breast cancer, colorectal and in prostate (Nelson et al., 2000).

Monitoring HER-2/neu extracellular domain that provides a tool for prognosis assessment, earlier detection of disease progression, predicting response to therapy, and timely intervention with an appropriate therapy (Carney et al., 2006). The EGFR family of receptor tyrosine kinases is an attractive target for antitumour strategies such as EGFR HER2/erb-B2 and HER3/erb-B3. Aberrant EGFR signaling is correlated with the progression of various malignancies. The somatic tyrosine kinase domain mutations in the EGFR gene are discovered in patients with non–small lung cancer cells corresponding to EGFR-targeted small molecular agents, such as erlotinib and gefitinib. EGFR overexpression is the principal mechanism of activation in various malignant tumours (Ono & Kuwano, 2006).

2.1.2(c) Monoclonal Antibody Therapy of Cancer

Although MAb in cancer treatment has resulted in new successes, new therapeutic and clinical challenges arise (Myskowski & Halpern, 2008). MAbs are important reagents in biomedical research for the purposes of targeted drug delivery systems, therapeutics and diagnostics of cancer, metabolic and hormonal disorders (Deb et al., 2013). MAb is used to block key receptors on tumour cell surfaces, as

well as compromising their functions. MAb is also used to recruit the cellular arm of the immune system and in planting a homing beacon on the transformed cell. Although MAbs are produced in the laboratory, they can mimic the naturally produced antibodies as part of the immune response to disease (Kewal et al., 2014).

According to the recent study, the MAb therapy for cancer has shown a remarkable advance, clarifying the advantage of MAb in treating common malignancies which include breast cancer, lymphoma, and colorectal cancers. Table 2.1 shows the antibody-based therapeutics which is applied in the therapies of human malignancies (Simpson & Caballero, 2014). Nowadays, tumour antigen (TA) targeted MAb has been among the successful new therapies. Clinical activity is observed as a single agent or in combination with radiotherapy or chemotherapy in metastatic colorectal cancer, follicular lymphoma, head and neck cancer, in addition to the breast cancer (Lee et al., 2011).

2.2 MONOCLONAL ANTIBODY IBMR3

The IBMR3 is a MAb with an IgM isotype. It is produced from 4 peptides, designated as number 2, 3, 4 and 5 as follows: peptide 2, S-E-N-D-P-A-D-F-R-I; peptide 3, W-S-E-W-S; peptide 4; F-V-S-V-G-P-T-Y-M-R-V-S; and peptide 5, M-K-V-L-Q-E-P-T-C-V-S-D-Y (Figure 2.2). Peptides 2, 3 and 5 represent sequences found on the extracellular domain while the peptide 4 represents sequence found in the intracellular portion of the human interleukin 4 receptor (IL-4R) at the C-terminus. They were chosen for their potential immunogenicity. Peptides 2, 3 and 5 are peptides from the N-terminal outside of the plasma membrane while peptide 4 is from the cytoplasmic portion of the molecule.

Table 2.1: Monoclonal antibodies approved for clinical use in oncology

Antibody name	Target	Antibody format	Application
Cetuximab	EGFR	Chimeric	Colorectal, breast and lung cancer
Panitumumab	EGFR	Human	Colorectal cancer
Nimotuzumab	EGFR	Humanized	Head and neck cancer
Rituximab	CD20	Chimeric	Non-Hodgkin lymphoma
Trastuzumab	HER2	Humanized	Breast cancer
Alemtuzumab	CD52	Humanized	Chronic lymphocytic leukemia
Bevacizumab	VEGFA	Humanized	Colorectal and lung cancer
Ofatumumab	CD20	Human	Chronic lymphocytic leukemia
Ipilimumab	CTLA-4	Human	Metastatic melanoma
Pertuzumab	HER2	Humanized	Breast cancer
Denosumab	RANK Ligand	Human	Solid tumour bony metastases
Brentuximab vedotin	CD30	Chimeric	Hodgkin's or systemic anaplastic large cell lymphoma
Gemtuzumabozogamicin	CD33	Humanized	Acute myelogenous leukemia
90Y-Ibritumomab tiuxetan	CD20	Mouse	Low grade or transformed B cell non-Hodgkin's lymphoma
Tositumomaband 131I-tositumomab	CD20	Mouse	Lymphoma

Adapted from Simpson and Caballero (2014)

MGWLCSGLLFPVSCLVLLRVASSGN**MKVLQEPTCVSDY**MSISTCEWKMNGPTNCSTELRLL

YQLVFLLSEAHTCVPENNGGAGCVCHLLMDDVVSADNYTLDLWAGQQLLWKGSFKPSEHV

KPRAPGNLTVHTNVSDTLLLTWSNPYPPDNYLYNHLTYAVNIW**SENDPADFRI**YNVTYLEPS

LRIAASTLKSGISYRARVRAWAQCYNTT**WSEWS**PSTKWHNSYREPFEQHLLLGVSVSCIVIL

AVCLLCYVSITKIKKKEWWDQIPNPARSRLVAIIIQDAQGSQWEKRSRGQEPAKCPHWKNCL

TKLLPCFLEHNMKRDEDPHKAAKEMPFQGSGKSAWCPVEISKTVLWPESISVVRCVELFEAP

VECEEEEEVEEEKGSFCASPESSRDDFQEGREGIVARLTESLFLDLLGEENGGFCQQDMGESC

LLPPSGSTSAHMPWDEFPSAGPKEAPPWGKEQPLHLEPSPPASPTQSPDNLTCTETPLVIAGNP

AYRSFSNSLSQSPCPRELGPDPLLARHLEEVEPEMPCVPQLSEPTTVPQPEPETWEQILRRNVL

QHGAAAAPVSAPTSGYQEFVHAVEQGGTQASAVVGLGPPGEAGYKAFSSLLASSAVSPEKC

GFGASSGEEGYKPFQDLIPGCPGDPAPVPVPLFTFGLDREPPRSPQSSHLPSSSPEHLGLEPGEK

VEDMPKPPLPQEQATDPLVDSLGSGIVYSALTCHLCGHLKQCHGQEDGGQTPVMASPCCGC

CCGDRSSPPTTPLRAPDPSPGGVPLEASLCPASLAPSGISEKSKSSSSFHPAPGNAQSSSQTPKIV

N**FVSVGPTYMRVS**

Figure 2.2: Amino acid sequences of human interleukin 4 receptor. The signal sequence and transmembrane domain are underlined, while the sequences used to synthesise peptides are indicated in bold letters (**SENDPADFRI** is the peptide 2; **WSEWS** is the peptide 3, **FVSVGPTYMRVS** is peptide 4 and **MKVLQEPTCVSDY** is the peptide 5). Adapted from Mat, 1992.

Peptide 5 is the outermost sequence on the N-terminal while peptide 3 represents the conserved WS x WS motif located outside the membrane-spanning domain (Mat, 1992). Amino acids codes for IL-4R showed in Table 2.2.

Table 2.2: Amino acids codes

One letter code	Amino acid
A	alanine
B	asparagine or aspartic acid
C	cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine
Z	glutamine or glutamic acid

2.2.1 Screening of IBMR3 Hybridoma Supernatants by Peptide ELISA.

The hybridoma supernatants were screened using ELISA technique for mice immunised with synthetic IL-4 receptor peptides. ELISA plates were coated with different peptides (2, 3, 4 and 5) and incubated with tissue culture supernatants from each of the wells that showed good cell growth. Cloned IBMR3 was derived from hybridoma cells and screened for immunoreactivity for peptide 3. This antibody was tested using ELISA coated with the other peptides (2, 3, 4 and 5). The antibody was found to be cross-reacted with all peptides.

IBMR3 antibody showed strong reactions with peptide 3, while peptide 5 recorded the highest OD. Even though there was similar concentration of different peptides used to coat the ELISA plates, the similarity of peptides binding efficiency was not determined. Peptide 3 and 5 might bind more efficient than peptide 2 and 4. Thus, higher OD405 was observed for peptide 3 and 5. There is possibility of different binding efficiency of different peptides on plastic, with peptide 5 being the most efficient. Since this antibody cross-reacted with all peptides, higher OD observed in peptide 5 might be due to the level of peptide bound rather than the inherent characteristics of the antibody (Mat, 1992).

2.2.2 The Expression of IBMR3 Supernatants

The supernatants were incubated with Jijoye cells (B lymphocyte). Resting and activated PBMC were also used. The antigen was weakly expressed by Jijoye cells and detected by IBMR3 antibody. This suggested that the antigen is expressed on the surface of Jijoye cells. However, the staining intensity was higher when the cells were fixed in acetone-methanol prior to immunostaining. Other than that, IBMR3 antibody showed weak positive result with resting PBMC, but expression

upregulation was observed in activated PBMC using anti-CD3 and IL-4. The supernatants were also incubated with frozen thymus sections. The IBMR3 antibody showed weak (1+) labelling for the thymic cortex, moderate (2+) labelling for the medullary region and strong (3+) reactivity for the endothelium of the medullary and interlobular blood vessels (Mat, 1992).

The IBMR3 antigen expression was analysed using flow cytometry on normal peripheral blood and leukaemic cells. The results showed no staining on the surface of normal resting PBMC, but high expression was observed at the cytoplasm of the cell. In the transformed haemopoietic cells, 9 acute myelogenous leukaemia (AML), 2 acute lymphoid leukaemia (ALL-T) and 6 ALL-B cells showed intense cytoplasmic staining and negative staining on the surface. The 2 AML cells showed staining in both cytoplasmic and surface regions (Hara & Mat, 2004). Previous study showed that IBMR3 antigen was expressed significantly higher at the cytoplasm of lymphocytes and monocytes when compared with their surface. The expression of IBMR3 antigen was differentially expressed in malignant haemopoietic cells (acute leukaemia) compared to normal PBMC (Hara, 2002).

2.2.3 Interleukin 4 and IBMR3 Monoclonal Antibody

The antibody supernatants were used in cross-linking study to determine the inhibition ability of IL-4 binding and/or immunoprecipitation of IL-4 receptor. Cells preincubation with IBMR3 tissue culture supernatants 10 minutes prior to the addition of ^{125}I-IL-4 did not block IL-4 binding. The antibody supernatants also did not immunoprecipitate the IL-4 receptor complex. These were preliminary experiment and more investigation on cross-inhibition and immunoprecipitation of

MAb IBMR3 and radiolabelled IL-4 needed to be done. It is to determine whether MAb IBMR3 recognises any component of human IL-4R complex (Mat, 1992).

When IBMR3 was added to the lymphocyte culture and incubated for one hour before adding the recombinant IL-4, the IBMR3 MAb failed to inhibit the binding of the human recombinant IL-4. Moreover, the experiment was done by incubating IBMR3 MAb concurrently with the recombinant IL-4 and the results showed increased proliferation of cell culture. This suggested that IBMR3 MAb has a low binding affinity as compared to IL-4. Therefore, IBMR3 MAb does not compete efficiently unless a "head start" is given. Alternatively, this antibody may bind to molecule involved in IL-4 signal transduction rather than the binding site (Mat, 1992).

2.3 INTERLEUKIN 4 RECEPTOR

Cytokines were discovered as secreted proteins that control various immune functions. It is now clear that cytokine functions extend to many other aspects of biology, including cancer (Lin & Karin, 2007). IL-4 is a cytokine which acts a critical role in the immune responses regulation. Its effect depends on the binding and signalling of a receptor complex consisting of IL-4Rα and gamma chains (γc), resulting in a series of phosphorylation mediated by receptor-associated kinases (Nelms et al., 1999). Binding of IL-4 activates signal transduction cascade including the Janus kinases of Jak1 and Jak3, which phosphorylate the cytoplasmic domain of the receptor as well as downstream signalling molecules. Two main pathways are activated in response to IL-4, signal transducer and transcription 6 (STAT6) activator pathway and the insulin receptor substrate-2 (IRS-2) pathway (Pernis et al., 1995).

IL-4 protects cells from apoptosis by 2 different pathways, one of them is mediated by IRS-1 (Zamorano et al., 1996).

IL-4 also acts as a multiunit transmembrane receptor (IL-4R) and induces growth inhibition in breast cancer cells (Gooch et al., 1998). This growth effect is associated with phosphorylation of 2 key components in the distinctive signalling pathways of STAT6 and IRS-1. The activation of these 2 pathways occurs independently in breast cancer cells (Gooch, Christy, & Yee, 2002). IL-4R is expressed as many as 30 % of primary breast tumour cells (Mat, 1992).

2.4 BREAST CANCER

Cancer is a disease of extreme heterogeneity. It is associated with the increase rate of death worldwide. Recent data showed that there were approximately 7.6 million deaths caused by cancer in 2008, attributing to 13 % death (Ferlay et al., 2010). In 2008, the cancer death in females was reported to be 1.38 million (23 %) cancer cases and 458,400 (14 %) cancer-related deaths (Jemal et al., 2011). The top 10 cancers affecting both male and female in Malaysia are breast, colorectal, lung, nasopharynx, cervix, lymphoma, leukemia, ovary, stomach and liver (Figure 2.3) (Zainal Ariffin & Nor Saleha, 2011).

Breast cancer is one of the common causes of cancer death among women (Jassem et al., 2013; Youlden et al., 2014) accounting for 18 % of all cases in 2012, and was the fourth most common cause of cancer-related deaths (9 %). The incidence rates rapid rises in recent years were observed in several Asian countries.

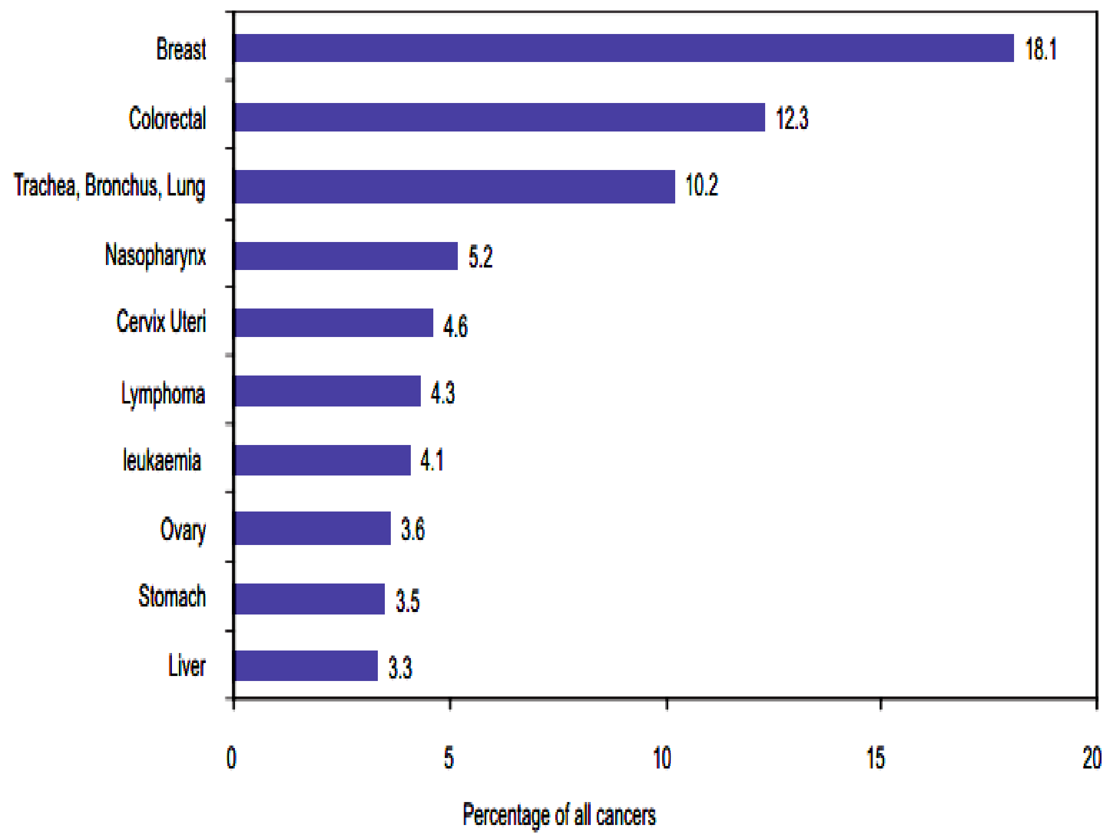

Figure 2.3: Ten most frequent cancers, all residence in Malaysia 2007. Adopted from Zainal Ariffin and Nor Saleha (2011).

Large increases in breast cancer mortality rates also occurred in many areas, particularly Malaysia (Youlden et al., 2014). Large increases in breast cancer mortality rates also occurred in many areas, particularly Malaysia (Youlden et al., 2014). The higher morbidity is linked to a late diagnosis of breast cancer's where cancer has reached the aggressive stages (Jassem et al., 2013). Breast cancer is dominant among Malaysian women aged between 40 and 49 (Chahil et al., 2015). Many studies showed that early detection of breast cancer had successfully led to various effective treatment options thereby saving life (Anderson et al., 2006).

The breast cancer treatment poses more restrictions to low and middle-income countries due to limited availability of trained medical personnel and modern equipment thus contributing to the high cost of cancer drugs and radiotherapy machines (Anderson et al., 2008). Rakha et al. (2010) highlighted the three main predictive determinants which are used in the routine practice of the early stage of breast cancer in which every patient has to be under a systemic therapy based on the conditions such as tumour size, lymph node status and histological tumour grade. The histological tumour grade depends on the degree of the tumour tissue differentiation. The pathologist has to observe the biology of histological grade on breast cancer behavior, which is essentially associated with the survival pattern (Rakha et al., 2010).

2.4.1 Prognostic Factors of Breast Cancer

Prognostic factors were used to evaluate an individual patient's risk of micrometastatic disease. The measurement accessible at the time of surgery which is associated with disease-free or overall survival in the absence of systemic adjuvant therapy is called a prognostic factor. This factor can be correlated with the history of

the disease (Cianfrocca & Goldstein, 2004). Prognostic factors must be distinguished from predictive factors. Predictive factors are useful in selecting the optimal adjuvant therapy for a particular patient. The predictive factor is a measurement that is related to response to a certain therapy (Clark, 2008). The most important determinants of 10-year survival for breast cancer patients are the conventional prognostic factors of survival such as tumour size, lymph node status, and grade (Soerjomataram et al., 2008).

2.4.2 Tumour Size

Tumour size is one of the most powerful breast cancer prognostic indicators. A larger tumour size has been associated with a more positive lymph nodes (Carter, Allen, & Henson, 1989; Weiss et al., 2003). Therefore, their interaction influences the survival from breast cancer. Nevertheless, the independence of survival against the node status is shown by the 66 % 10-year survival rate found in node negative patients with a tumour of 2–5 cm as compared to 79 % in those with a tumour smaller than one cm (Chia et al., 2004).

2.4.3 Axillary Lymph Node Status

The histological presence of axillary lymph node is an important factor in breast cancer prognosis (Elkhodary et al., 2014). Patients with positive lymph node were reported to have a 4 to 8 times higher mortality rate in comparison to patients with negative lymph nodes (Stankov et al., 2012). The biomarker expression in lymph node metastasis provides prognostic information when no primary analysis tumour can be done. Therefore, a treatment selection based on biomarkers in the lymph node a topic to be studied further (Falck et al., 2010).

2.4.4 Histologic Grade

Tumour grade has prognostic significance and is initially used to make decisions on lymph node negative patients with borderline tumour size (Cianfrocca & Goldstein, 2004). According to the study conducted by Pilli and Godhi (2013), histologic grade refers to the degree of tumour differentiation. Any tumour grade is based on the tumour malignancy degree which is reflected in the morphological structure. The histological grade in breast cancer provides clinically important prognostic information (Sotiriou et al., 2006). In grade one, the cancer cells like normal cells and they are growing slowly. It is called well-differentiated or low grade. Grade 2 which the cancer cells are not like normal cells. They are growing faster. It is called moderately differentiated or intermediate/moderate grade. Grade 3 which the cancer cells look totally different than the normal cells. It is called poorly differentiated or high grade (www.breastcancer.org, 2006).

The tumour mitotic rate and the occurrence of abnormal mitoses are the important characteristics (Bignold, Coghlan, & Jersmann, 2006). According to Rakha et al. (2010) when histological grading is carried out, it provides a simple, inexpensive, and highly accurate method for assessing tumour biological characteristics and patient prognosis. This is important for breast cancer patients in the world where access to a new molecular technology is not currently available.

The Scarff-Bloom-Richardson (SBR) grading is one of the important pathological parameters to be evaluated in the management of breast carcinoma. The SBR grade is a useful and sensitive guide for selecting adjuvant systemic therapy and the method should be standardized for cytology specimens (Elston & Ellis, 1991).

2.4.4(a) Scarff-Bloom-Richardson Grading System

Bloom and Richardson proposed a simplified system that utilised only three of Greenough's variables: gland-formation (tubularity), degree of variation in nuclear size and shape (pleomorphism) and 'hyperchromatic figures' as estimation for proliferation (Meyer et al., 2005). For each case, the SBR tumour grading was done on fine-needle aspirates and tissue sections by two independent pathologists. The results were reproducible in the majority of the cases. The discrepant findings were discussed and a final consensus was agreed upon the usage of multiheaded microscope. Each of the three features, i.e. tubule formation, nuclear pleomorphism and mitotic count were scaled as 1, 2 or 3 and the final SBR score ranged between 3 – 9, which was further divided into three grades (I – III). For grade I, the score ranged between 3 – 5, for grade II the score was 6 – 7 and for grade III the score ranged between 8 – 9. Cytology criteria for identifying tubule formation and mitotic counts were modified from the standard histological criteria. Scaling of nuclear pleomorphism on cytology was similar as in tissue sections. Tubule formation in FNA smears were identified as microacini and/or as branching, elongated, three-dimensional tubular structures (Bansal et al., 2012).

2.4.5 Triple Negative Breast Cancer

Triple negative breast cancer (TNBC) is a heterogeneous disease which is associated with poor prognosis and has a lack of expression of the estrogen receptor (ER), progesterone receptor (PR) and epidermal growth factor receptor type 2 (HER2) (Millis et al., 2015).