COMPARISON OF IBMR3 ANTIGEN EXPRESSION IN NORMAL AND MALIGNANT BREAST TISSUES (TISSUE MICROARRAY)

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ABSTRACT:

Breast cancer is the most common cancer in women in most part of the world. It is also the most common cause of cancer death, still carrying a high morbidity and mortality. Advances in the understanding of tumor biology have led to the development of targeted therapies allowing progress in breast cancer treatment. Monoclonal antibodies (MAb) have been widely used in medicine for research, diagnostic and therapeutic purposes. The aim of this study is to determine the IBMR3 antigen expression in normal and cancer tissues of the human breast and clinicopathological parameters. In this study, indirect immunofluorescence method was performed on tissue microarray slides which included 2 cases of normal breast tissues and 59 cases of breast cancers. Positive immunofluorescent staining for IBMR3 expression was detected in 2/2 of normal breast tissues and 59/59 of breast cancer. There was no significant number of breast cancer tissues expressing the IBMR3 antigen as compared to normal breast tissue. However, there are differential expression of the IBMR3 antigen in the cancer tissues with (59.3%) showing weak expression, (28.8%) moderate and (11.9%) strong reaction. Normal tissues show moderate and strong expression of the antigen.

This result may indicate that the degree of expression of the antigen may be associated with the biology of the cancer cells. However, we

cannot perform any correlation study since the supplier of the micro array tissues did not provide the grading of the cancer tissue.

Key words: TMA (Tissue microarray) – IBMR3 – Indirect Immunofluorescence. Breast tissue

الملخص:

يعد سرطان الثدي أكثر أنواع السرطان شيوعًا بين النساء في معظم أنحاء العالم. وهو أيضًا السبب الأكثر شيوعًا للوفاة بالسرطان، ولا يزال يحمل نسبة عالية من القدرة على إحداث المرض والوفيات. أدى التقدم في فهم بيولوجيا الأورام إلى تطوير علاجات ساهمت بإحراز تقدم في علاج سرطان الثدي.

تم استخدام الأجسام المضادة وحيدة النسيلة (MAb) على نطاق واسع في الطب لأغراض البحث والتشخيص والعلاج. الهدف من هذه الدراسة هو تحديد تعبير مستضد IBMR3 في الأنسجة الطبيعية والسرطانية للثدي البشري والعلامات المرضية السريرية. في هذه الدراسة، تم إجراء طريقة (indirect immunofluorescence) على شرائح الأنسجة (tissue microarray) والتي شملت حالتين من أنسجة الثدي الطبيعية و59 حالة من سرطان الثدي. تم تسجيل صبغ إيجابي(immunofluorescence) للمستضد IBMR3 في 2/2 من أنسجة الثدي الطبيعية و59/59 من سرطان الثدي. لم يكن هناك اختلاف واضح لتعبير مستضد IBMR3 في أنسجة سرطان الثدي مقارنة بأنسجة الثدي الطبيعية. ومع ذلك، هناك تعبير تفاضلي للمستضد IBMR3 في الأنسجة السرطانية حيث أظهر (59.3%) تعبيراً ضعيفاً، (28.8%) رد فعل متوسطاً و(11.9%) رد فعل قوياً. تظهر الأنسجة الطبيعية تعبيرًا معتدلًا وقويًا عن المستضد.

قد تشير هذه النتيجة إلى أن درجة التعبير عن المستضد قد تكون مرتبطة بالتركيب البيولوجي لخلايا السرطانية. ومع ذلك، لا يمكننا إجراء أي ارتباط نظرًا لأن البيانات لأنسجة المصفوفة الدقيقة لا تحتوي على تصنيف الأنسجة السرطانية من المصدر.

الكلمات المفتاحية: TMA (مصفوفة الأنسجة الدقيقة) – IBMR3 – indirect immunofluorescent - أنسجة الثدي.

1. INTRODUCTION:

Over the years, health-related issues, such as cancer have attracted global attention. A quite number of medical researchers, practitioners, scholars, policy and decision-makers as well as planners and administrators have being bordered with diagnostic, curative, and

preventive activities relating to cancerous diseases on one hand and some have even fallen prey of these diseases on the other. It is out of these worries that discovery was made about cancer as the second leading cause of death for both men and women in the world and it is expected to become the leading cause of death in the next few decades (Tang *et al*, 2009).

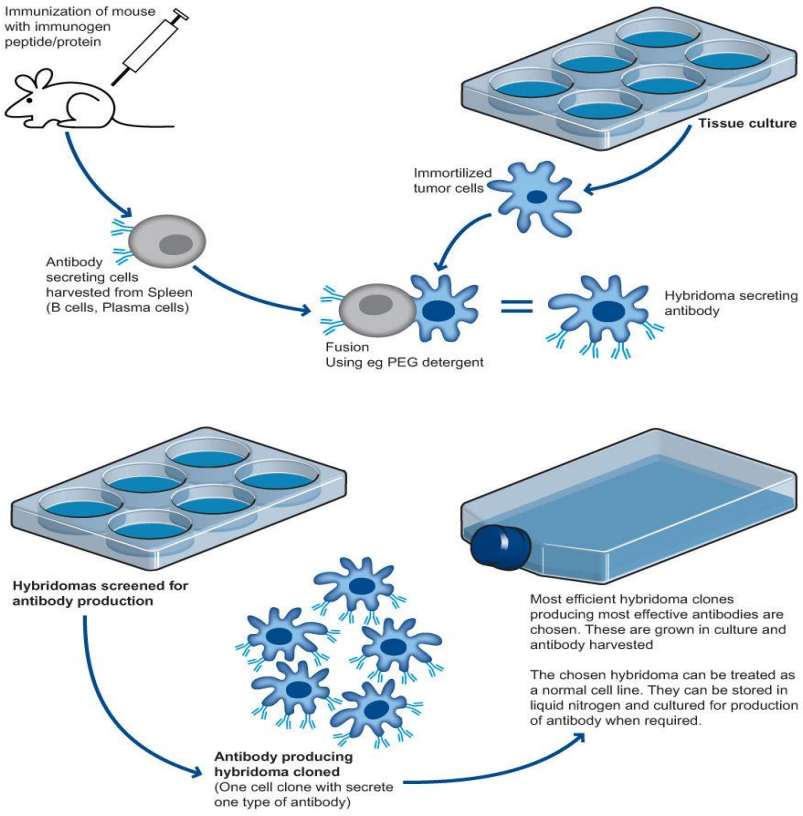
The main aim of the study centered on a comparative analysis of IBMR3 antigens expression in normal and malignant breast cancer tissues, specifically microarray among women. It is out of this broad aim that the two (2) specific objectives of the study came up. The research objective therefore seeks to:

- Examine the IBMR3 antigen expression in normal tissues of women's breasts.
- Explore the IBMR3 antigen expression in cancer tissues of women's breasts.

Monoclonal antibodies (MAbs) have been widely used in medicine for research, diagnostic and therapeutic purposes (Mat *et al*, 2007). Monoclonal antibodies are well-defined antibodies. They also have known as monospecific antibodies with reproducible characteristics. The variety and composition of these types of antibodies vary from animal to animal. Hybridoma technology has ever being used for the generation of monoclonal antibodies. In addition, they are useful in target specific therapy against dangerous diseases. This property of monoclonal antibody offers us a new hope to combat deadly diseases where chemotherapy fails to produce specific treatment without adverse effects (Dirk, 2002) .The development of monoclonal antibodies (MAbs) technology was a milestone in the field of biomedical science. The first reports of monoclonal antibodies (MAbs) production were made by Köhler and Milstein in 1975, when they demonstrated that monoclonal antibodies (MAbs) could be produced by fusing antigen specific antibody-producing murine B-

lymphocytes with an immortalized myeloma cell line (Kohler & Milstein, 1975).

IBMR3 is a monoclonal anti body produced by Mat and others in 1992 in mouse. Monoclonal antibody IBMR3 (MAb IBMR3) was generated against synthetic peptides of the published amino acid sequences of the human interleukin-4 receptor (hIL-4R) with an initial intention to further study the role of interlukin-4 (IL-4) and its receptor in tumourigenesis (Hara *et al*, 2004).



Hybridoma antibody production

Figure: Schematic of the monoclonal antibody production procedure. (www.abcam.com/technical)

2.2 Tissue Microarrays (TMAs):

The tissue formalin-fixing, paraffin-embedding, sectioning technique was invented in 1850s. This method only allows on tissue in

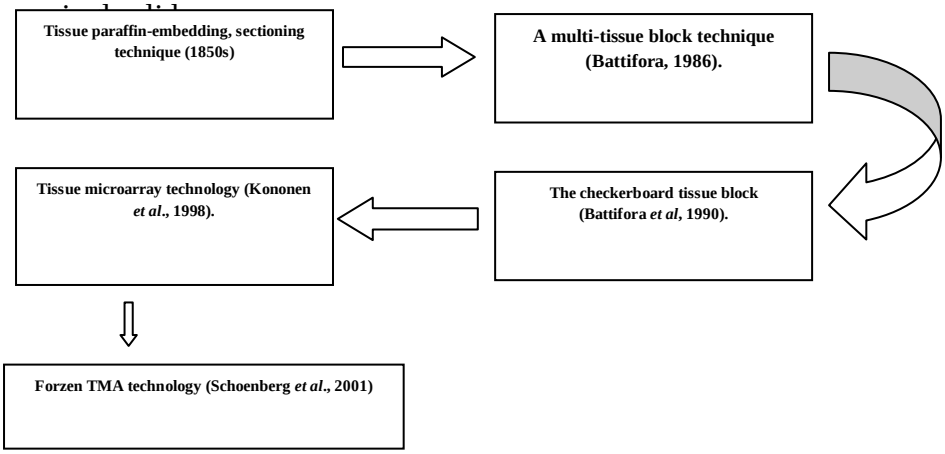


Figure: 2. The history of TMAs

Tissue microarrays (TMAs) are new tools consisting of miniaturized collection of arrayed tissue cores (diameter 0.6 mm) on a microscope glass slide, that allow for high-throughput expression profiling of tissue samples. Different techniques could be employed for identification of specific phenotypic (immunohistochemistry and in situ hybridization) or genotypic (fluorescence in situ hybridization) alterations (Hayat, 2005).

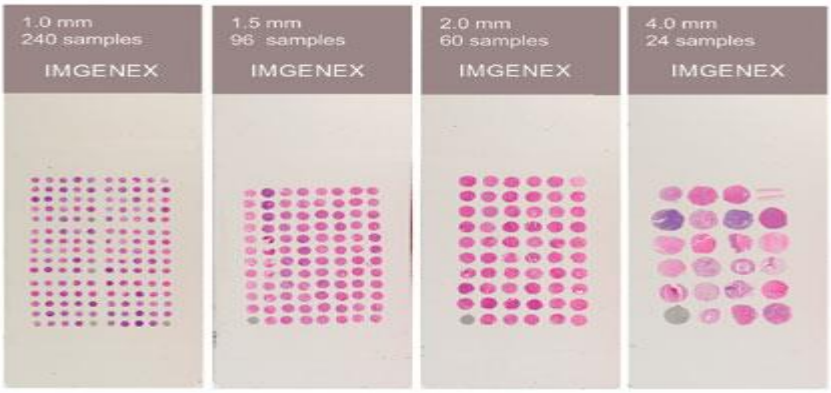


Figure: 2.5. Tissue microarray (www.nature.com/.../v3/n7/full/nmeth0706-571.html)

3 - MATERIALS AND METHODOLOGY

3.1 Materials and reagents

The materials, which were used in this study are adjustable pipettes, cover slips (24 mm x 60 mm), slides, staining gar, fume hood, universal hot air oven, stopwatch, fluorescence microscope, beakers and reagents listed in Table 3.1.

3.2 Tissue microarray samples

Normal breast tissue microarray and breast cancer were purchased from the Biomax, USA. The slide (two items) was contained 63 cases, 3 cases of normal breast tissue and 60 cases of breast cancer tissues.

3.3 Positive and negative control

The positive control for IBMR3 was anti-cytokeratin diluted 1:40 with phosphate buffer saline (PBS). Phosphate buffer saline was used as negative control.

3.4 Tissue preparation for negative control

The breast cancer tissues were obtained from Hospital Seberang Jaya with ethical approval. However, frozen sectioning process was performed by microtome (Cryostat MTE). The thickness for each tissue was six microns. Then the slides incubated for 10 minutes at -20^{o} C with methanol.

Table: 3.1 Reagents

No.	Reagent	Manufacturer	Catalogue No.
1	Primary antibody (IBMR3)	Available	
2	Breast tissue microarray	US Biomax	BC08016
3	Phosphate buffer saline (PBS), pH7.0	Sigma	MFCD00131855
4	Polyclonal Rabbit Anti-Mouse Immunoglobulins/FITC (Secondary Antibody)	Dako	F0232
5	Anti-cytokeratin (Positive control)	Dako	M3515
6	Alcohol	Sigma	
7	Xylene	Sigma	

3.5 Indirect Immunofluorecent [IIF]

3.5.1 Deparaffinization

Firstly, tissue microarray slides were deparaffinized by incubating the slides in oven at 60^{o} C for one hour. The slides were twice immersed in xylene for 10 minutes. Subsequently, the slides were immersed in absolute ethanol for 2x2 minutes and immersed in different concentration of ethanol as following:

Ethanol 95% for 2 minutes than ethanol 95% for 2 minutes than ethanol 80% for 2 minutes. Thereafter, the slides were rinsed in distilled water twice.

3.5.2 Technique indirect immunofluorecence for IBMR3 antigen

Briefly, indirect immunofluorecence technique was applied after deparaffinization; the negative control slide rinsed in phosphate buffer saline PBS 5x3 minutes. Subsequently, the slides were incubated in primary antibody for 60 minutes (anti-cytokeratin diluted with PBS 1/100) at room temperature. Later, the slides were rinsed in phosphate

buffer saline (PBS) 3x5 minutes and incubated in polyclonal rabbit anti-mouse immunoglobulins/fluorescein-isothiocyanate (FITC) secondary antibody (apply in a darkened room) (Dako, USA) for 30 minutes. Later, the slides were rinsed in PBS for 3x5 minutes. Than the slides were mounted with glycerol and with cover slips. Thereafter, the slides were observed under fluorescent microscope (Leica).

3.5.3 Interpretation of indirect immunofluorecence

Positive staining is characterized by bright green. The stain is graded by two independent assessor (pathologist and student) according to a scale of 0 (negative staining), 1+ (weak staining), 2+ (moderate staining) and 3+ (strong staining).

3.5 Statistical analysis

After indirect immunoflurecence analysis of all breast cancer and normal human breast tissue slides, data were recorded and analyzed statistically. Chi Square test was used to determine if there are any differences between expression level of this antigen (IBMR3) in breast cancer and normal breast. The relationship between this antigen expression in breast cancer and patient's age and other clinical data were also analyzed. Statistical analysis of the data is performed using SPSS (Statistics Package for Social Science, version 12.0) software.

4 - RESULTS

4.1 Description of the demographic characteristics of the patients implicated in the TMA

The study considered two TMA slides. The Slide BC08016 consists of 2 cases of normal breast tissue, 1 case breast adenosis, 4 cases of intraductal carcinoma with early infiltrating, 1 case of medullary carcinoma, 1 case of blood vessel and fatty tissue, 1 case of fibro fatty tissue, 52 cases of infiltrating duct carcinoma and 2 samples were lost during data processing.

4.1.1 Age group

Each core is representing one case/patient. The mean age was 40.3 years. The age of the cases varies between 21 to 72 years. For normal breast tissue, one case was 49 years and other case was 50 years. For breast cancer tissue, 33 cases (55.9%) were between 20-39 years, 20 cases (33.9%) were between 40-59 years and 6 cases (10.2%) were between 60-79 years. Therefore, majority of the cases fall between 20-40 years age group. See Figure 4.1 below.

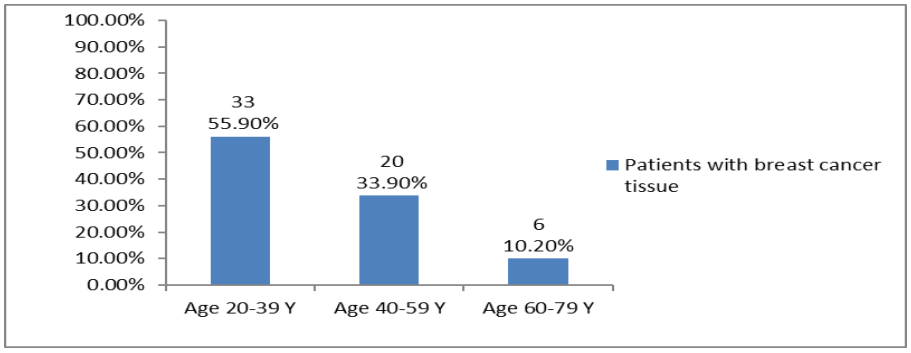


Figure 4.1 Distribution of patients with breast cancer tissue by age category

4.1.2 Types of breast cancer

Most of the cases (52 cores) were diagnosed with infiltrating duct carcinoma (88.1%), and 4 cases (6.8%) represented infiltrating carcinoma with early infiltrating, while only 1 case (1.7%) represented blood vessel and fatty tissue. The medullary carcinoma was represented by 1 case (1.7%) and fibrofatty tissue had only 1 case (1.7%) representation. See Figure 4.2 below:

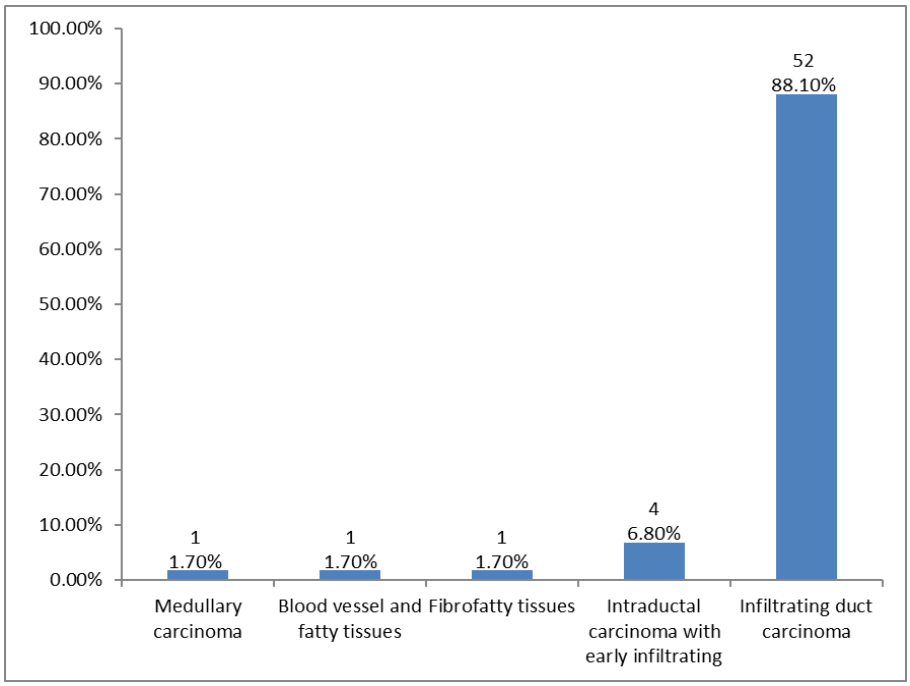


Figure 4.2 Distribution of the types of breast cancer

4.3 Immunofluorescent Staining for IBMR3 in tissues

4.3.1 Positive and negative control

The positive control used is anti-cytokeratin, which was diluted with phosphate buffer saline, 1:100. As for negative control, phosphate buffer saline was used instead of primary antibody. See Figure 4.3 below.

4.3.2 IBMR3 expression in normal and breast cancer tissues

4.3.2.1 Intensity of staining

The intensity of staining of IBMR3 in normal breast tissue was moderate (++) for one case (50%) shown , strong (+++) for one case (50%) and one case was lost during processing. IBMR3 expression in breast cancer tissue was weak (+) for (35/59) cases (59.3%), moderate for (17/59) cases (28.8%) and strong for (7/59) cases (11.9%). See Figures 4.4 – 4.6.

The chi-square test showed that there was no significant number of breast cancer tissues expressing the IBMR3 antigen as compared to normal breast tissue (P = 0.168) (Table 3.1). High percentage of normal breast tissue appeared to have high expression IBMR3 (+++, strong staining) (50%) as compared to breast cancer tissue (11.8%). In addition, normal breast tissue appeared to have high expression IBMR3 (++, moderate staining) (50%) as compared to breast cancer tissue (28.8%).

Table 4.1 Summary of intensity of the staining results obtained following analysis of normal and breast cancer tissue for IBMR3

1 Weak staining

	Sample size (n = 61)	IBMR3			*P* value
		1	2	3	
Normal breast	**2**	**0**	**1 (50%)**	**1 (50%)**	**0.168**
Breast cancer	**59**	**35 (59.3%)**	**17 (28.8%)**	**7 (11.8%)**	

2 Moderate staining

3 Strong staining

A B

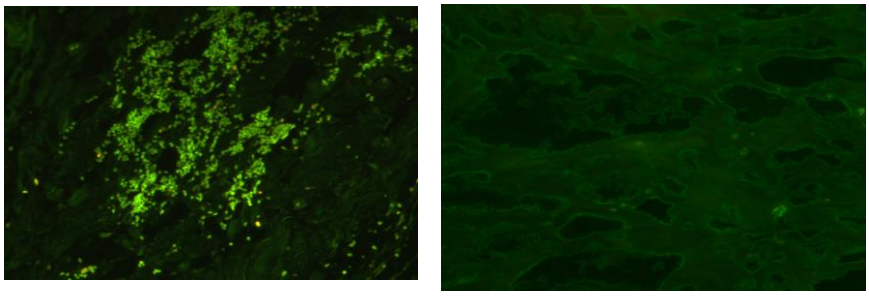


Figure 4.3 The figure above represented immunofluorescent staining for positive and negative control in breast cancer tissue. The (A) represented the positive control using anti-cytokeratin magnification X10, while the (B) represented negative control using phosphate buffer saline magnification X20.

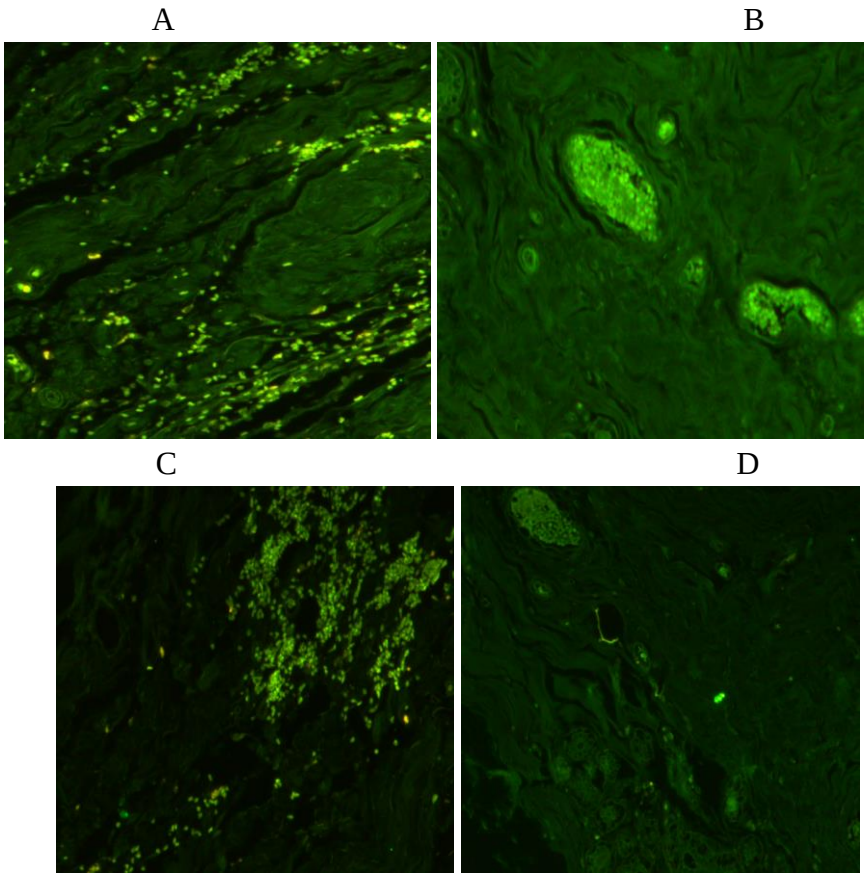


Figure 4.4: The figure above represented the immunofluorescent staining for same normal breast tissue (A) strong staining of IBMR3 magnification X20, and (B) moderate stainig of IBMR3 magnification X20 as well as (C) strong staining of anti-cytokeratin magnification X10 (D) and moderate staining of anti-cytokeratin magnification X20.

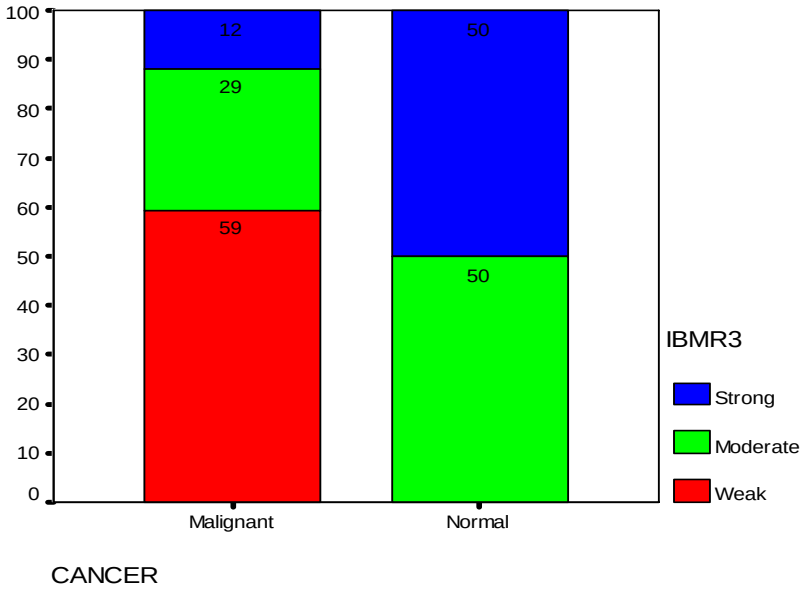
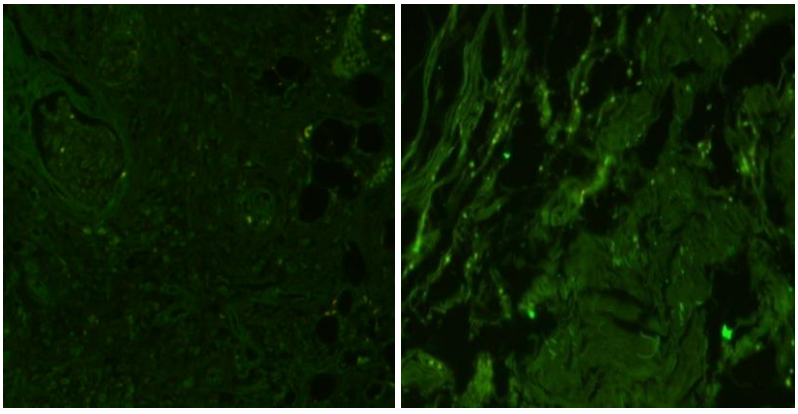


Figure 4.5 **Distribution of IBMR3 positive cases in normal and breast cancer tissues.**

A B



C

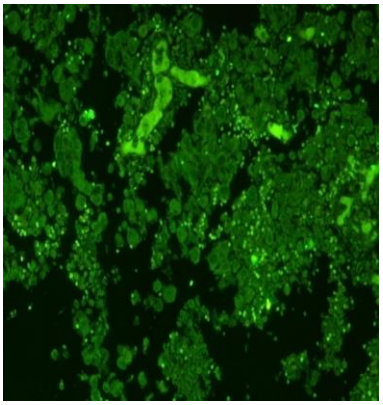


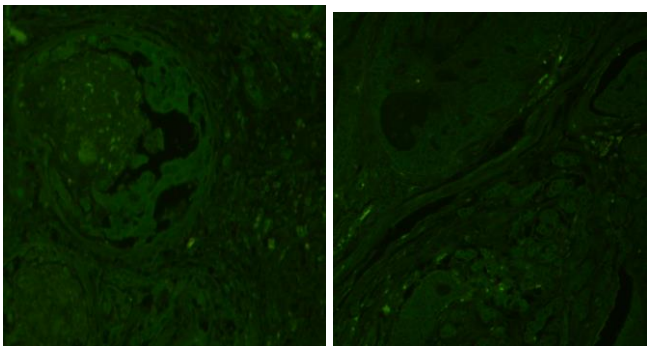
Figure 4.6 The three figures; A, B, and C above represented the immunofluorescent stained IBMR3 in breast cancer tissue. The figure A showed a weak immunofluorescent stained IBMR3 in breast cancer tissue magnification X20, figure B captures a moderate immunofluorescent stained IBMR3 in breast cancer tissue magnification X20, while the figure C demonstrates strong immunofluorescent stained IBMR3 in breast cancer tissue magnification X10.

4.3.2.2

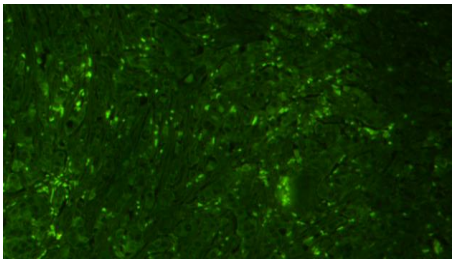
4.3.2.3 Expression of IBMR3 and types of breast cancer tissues

Each of the infiltrating duct carcinoma showed weak staining for 32 cases (61.5%), moderate staining for 15 cases (28.8%) and strong staining for five (5) cases (9.7%). The medullary carcinoma showed weak staining for one case (100%). The fibrofatty tissue showed strong staining for one case (100%). Blood vessel and fatty tissues showed strong staining for one case (100%), whereas the intraductal carcinoma with early infiltrating showed weak staining for 2 cases (50%), and moderate staining for 2 cases (50%). See Figures 4.7 to 4.11 below

A B

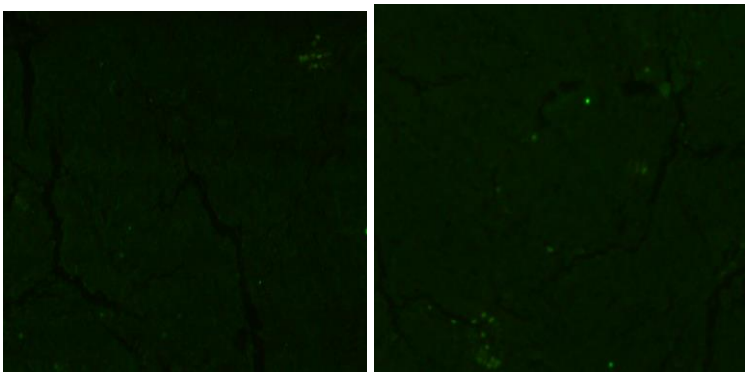


C



The Figure 4.7: A, B and c above gave the representations of the immunofluorescent staining of IBMR3 in different infiltrating duct carcinoma. The A presented a weak staining magnification X20, while B represented a moderate staining magnification X20 and C captured strong staining magnification X20.

A B



C D

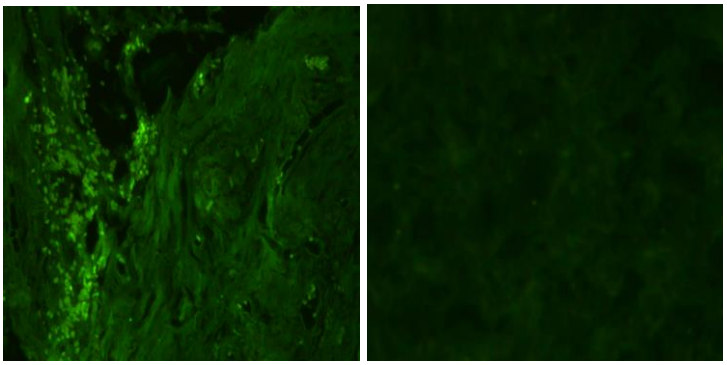
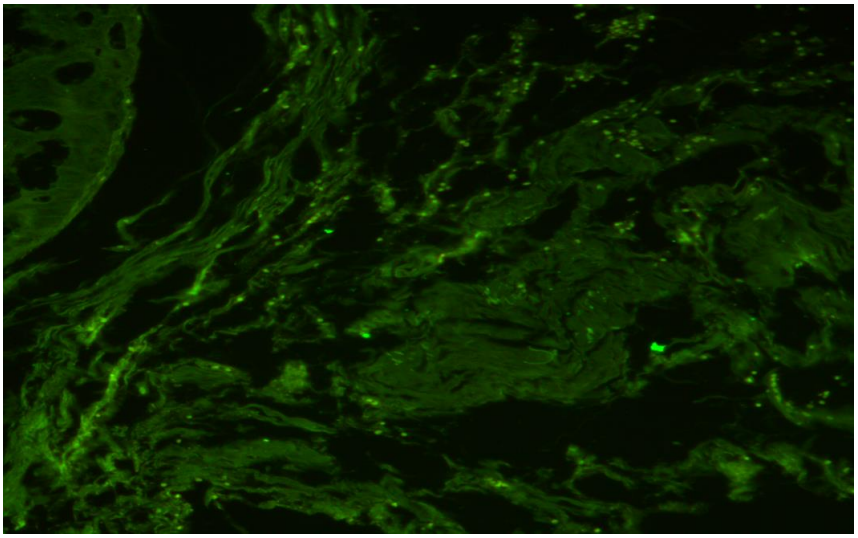


Figure 4.8: This represented immunofluorescent staining for: (A) weak staining of IBMR3 in medullary carcinoma magnification X20, (B) weak staining of anti-cytokeratin in medullary carcinoma magnification X20, (C) strong staining of positive control magnification X20and (D) staining of negative control magnification X20.

A



B

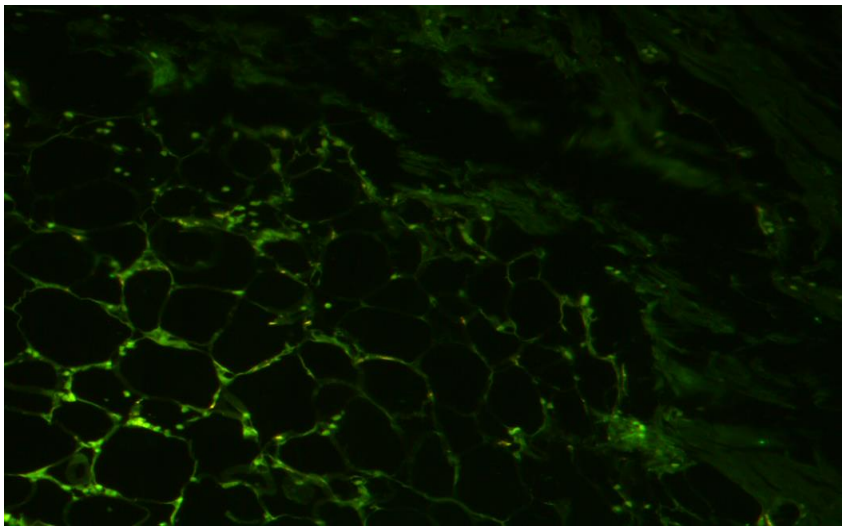


Figure 4.9: The figure above represented immunofluorescent staining for (A) strong staining of IBMR3 in fibrofatty tissue magnification X20 and (B) strong staining of anti-cytokeratin in fibrofatty tissue magnification X20.

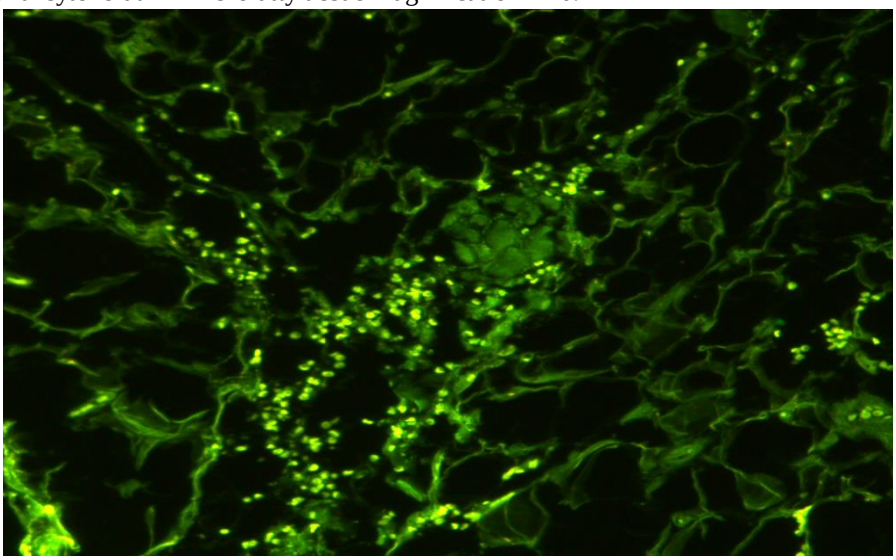
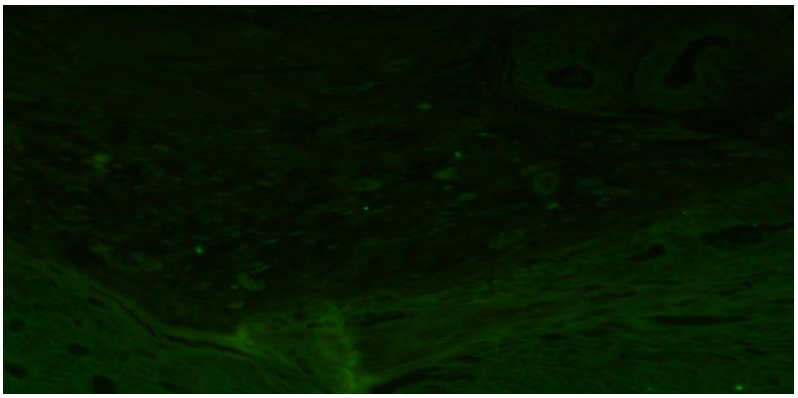


Figure 4.10: The figure 4.10 above represented immunofluorescent staining for strong

Staining of IBMR3 in blood vessel and fatty tissue magnification X20.

A



B

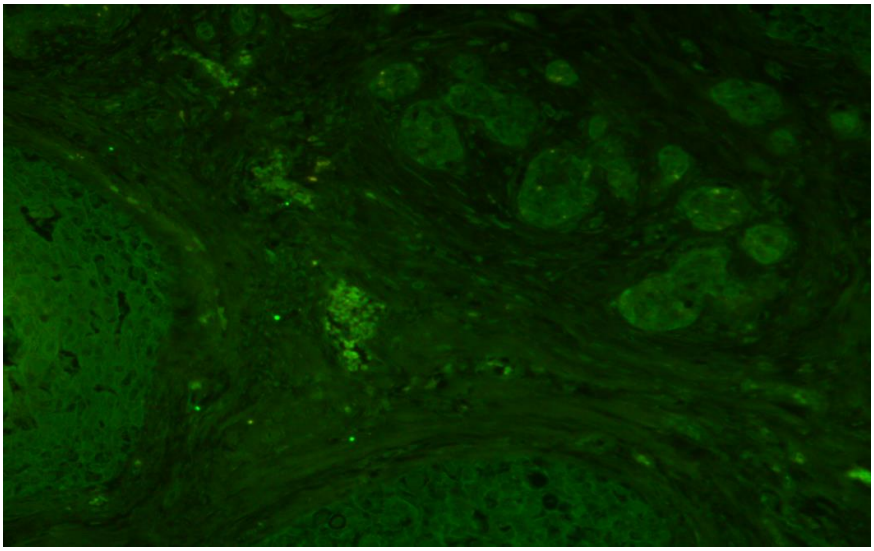


Figure 4.11: The figure 4.11 above represented Immunofluorescent staining of IBMR3 in intraductal carcinoma with early infiltrating for (A) weak staining magnification X20 and (B) moderate staining magnification X20.

4. 3.2.3 Expression of IBMR3 and age group

In the normal breast, the intensity of staining of IBMR3 was moderate for 1 case and strong for 1 case in patients between 40-59 years age group. In breast cancer tissues, the staining was weak for 19 cases, moderate for 9 cases and strong for 5 cases in patients between 20-39 years age group. Between 40-59 years, the staining was weak for 12 cases, moderate for 7 cases and strong for 1 case. Between 60-79 years, the staining was weak for 4 cases, moderate for 1 case and strong for 1 case. There are no significant relationship between IBMR3 expression and age group (Table 4.2).

Table 4.2 Summary of the results obtained following analysis of IBMR3 expression and age of patients

		Sample size (n= 61)	1	2	3	*P* value
Normal breast tissues	20-39	0	0	0	0	0.249
	40-59	2	0	1 (50%)	1 (50%)	
	60-79	0	0	0	0	
Breast cancer tissues	20-39	33	19 (57.5%)	9 (27.3%)	5 (15.2%)	
	40-59	20	12 (60%)	7 (35%)	1 (5%)	
	60-79	6	4 (66.6%)	1 (16.7)	1 (16.7)	

1 Weak staining

2 Moderate staining
3 Strong staining

5 - DISCUSSION

IBMR3 is a monoclonal anti body produced by Mat et at (1992) in mouse. It is believed, that this is the first study to analyze IBMR3 antigen expression in breast cancer using tissue microarray (TMA). This preliminary study of IBMR3 antigen expression was determined using TMA. It included 3 cores of normal breast tissues and 60 cases of breast cancer tissues. However, there were 1 normal breast tissue and 1 breast cancer tissue which were not included in the statistical analysis. Therefore, only 2 cases normal breast tissues and 59 cases of breast cancer tissues were used for further analysis. Indirect immunofluorecence method was carried out to detect the expression of IBMR3 in both normal breast tissues and breast cancer tissues. This study has therefore found that, positive indirect immunofluorecence staining for IBMR3 expression was detected in 2/2 of normal breast tissues and 59/59 of breast cancer. However, there are differential expression of the IBMR3 antigen in the cancer tissues with (59.3%) showing weak expression, (28.8%) moderate and (11.9%) strong reaction. Normal tissues show moderate and strong expression of the antigen. In addition, the study compared the expression of IBMR3 antigen in the tissues based on the age of cases. During the comparison, it appeared that there were no correlation between the age and the expression of IBMR3 antigen, whereas, from the only single previous study under review (Hara *et al*, 2004) it was indicated that, IBMR3 antigen appeared in the immunohistochemical analysis of breast tissues. Thus, the results revealed strong IBMR3 antigen expression in malignant tissues, but not in normal counterpart from the same patients, suggesting that IBMR3 antigen can be expressed differentially in normal and malignant breast tissues (Hara *et al*, 2004). However, some obstacles were faced during the preparation to

this preliminary study. The difficulties faced included shortage of sample size of normal breast tissues due to the tissue micro-arrays were not provided by the first company. This limited the experimental time. More so, few texts and indeed literature limited the scope of the research topic, which led to inadequate information about the IBMR3 antigen references. This shortage in the reference materials occurred due to the novel study related to IBMR3 antigen still under research. The inadequate access to tissue micro-arrays also posed serious defect to the study. All the factors mentioned above limited the capacity in the interpretation of data and discussion of results in the study.

In conclusion, it was revealed that IBMR3 antigen expression has various grades in breast cancer tissues. These findings therefore suggest that variations in IBMR3 antigen expression are bound to be experienced due to extraneous factors, which include the stage of breast cancer, tumor biology, age, and clinical status.

In spite of the results obtained from this study, we recommend that further investigation with larger sample size should be conducted in order to confirm and uncover the associations being investigated in this study.

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APPENDIX

Tissue microarray (IBMR3 and Anti-cytokeratin) expression results

Pos	No	Sex	Age	Organ	Pathology diagnosis	Type	Anti-cytokeratin expression	IBMR3 expression
A1	1	F	45	Breast	1	Malignant	+	+
A2	2	F	51	Breast	1	Malignant	++	++
A3	3	F	58	Breast	1	Malignant	+	+
A4	4	F	21	Breast	1	Malignant	++	+
A5	5	F	25	Breast	1	Malignant	+	+
A6	6	F	61	Breast	1	Malignant	+	+
A7	7	F	38	Breast	1	Malignant	+++	+++
A8	8	F	72	Breast	1	Malignant	++	++
A9	9	F	27	Breast	1	Malignant	+	++
B1	10	F	52	Breast	1	Malignant	++	+
B2	11	F	28	Breast	2	Malignant	+	+
B3	12	F	28	Breast	1	Malignant	+++	+++
B4	13	F	28	Breast	1	Malignant	+	+
B5	14	F	28	Breast	1	Malignant	++	++
B6	15	F	28	Breast	1	Malignant	++	+
B7	16	F	41	Breast	1	Malignant	+	+
B8	17	F	40	Breast	1	Malignant	+	+
B9	18	F	50	Breast	3	Malignant	++	+++
C1	19	F	35	Breast	1	Malignant	+++	+++
C2	20	F	41	Breast	1	Malignant	+	+
C3	21	F	48	Breast	2	Malignant	+++	++
C4	22	F	64	Breast	1	Malignant	+	+
C5	23	F	29	Breast	1	Malignant	+	++
C6	24	F	29	Breast	1	Malignant	+	+
C7	25	F	56	Breast	1	Malignant	++	+
C8	26	F	40	Breast	1	Malignant	+	+
C9	27	F	48	Breast	1	Malignant	+	+
D1	28	F	30	Breast	1	Malignant	+	+
D2	29	F	30	Breast	1	Malignant	++	+
D3	30	F	30	Breast	1	Malignant	+++	++
D4	31	F	57	Breast	1	Malignant	+	+
D5	32	F	56	Breast	1	Malignant	+	++
D6	33	F	62	Breast	1	Malignant	+	+
D7	34	F	47	Breast	1	Malignant	++	++
D8	35	F	30	Breast	1	Malignant	+	+
D9	36	F	42	Breast	1	Malignant	+	+
E1	37	F	30	Breast	2	Malignant	+++	++

E2	38	F	30	Breast	1	Malignant	+	+
E3	39	F	30	Breast	1	Malignant	+	++
E4	40	F	30	Breast	2	Malignant	+	+
E5	41	F	30	Breast	1	Malignant	+	+
E6	42	F	64	Breast	1	Malignant	+	+
E7	43	F	31	Breast	1	Malignant	+	++
E8	44	F	31	Breast	1	Malignant	+	+
E9	45	F	31	Breast	1	Malignant	+	+
F1	46	F	49	Breast	1	Malignant	+	+
F2	47	F	31	Breast	1	Malignant	+	+
F3	48	F	31	Breast	1	Malignant	++	++
F4	49	F	31	Breast	****	Malignant	****	****
F5	50	F	58	Breast	1	Malignant	+	++
F6	51	F	32	Breast	1	Malignant	++	+++
F7	52	F	45	Breast	1	Malignant	+++	++
F8	53	F	32	Breast	1	Malignant	+	+
F9	54	F	72	Breast	1	Malignant	+++	+++
G1	55	F	32	Breast	4	Malignant	+	+
G2	56	F	32	Breast	1	Malignant	+	+
G3	57	F	50	Breast	1	Malignant	+	++
G4	58	F	32	Breast	1	Malignant	+	++
G5	59	F	32	Breast	5	Malignant	++	+++
G6	60	F	32	Breast	1	Malignant	+	+
G7	61	F	49	Breast	6	Normal	++	+
G8	62	F	50	Breast	6	Normal	+	****
G9	63	F	50	Breast	6	Normal	+++	+++

1 Infiltrating duct carcinoma
2 Intraductal carcinoma with early Infiltrating
3 Fibrofatty tissue
4 Medullary carcinoma
5 Blood vessel and fatty tissue
6 Normal breast tissue
\+ Weak staining
++ Moderate staining
+++ Strong staining
**** Lost tissue